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(54) Title: DEVELOPMENT OF REGULATORY CELLS AS A MEANS FOR TREATING AUTOIMMUNE DISEASE

(57) Abstract

A population of mammalian T–cells enriched in cells which have a cytokine production profile characteristic of Th2–like cells (IL–4 production, etc.) is produced by culturing T–cells, including thymocytes, in a conditioned medium (CM), which is prepared by culturing mammalian cells such as peripheral blood cells in the presence of plant mitogens. The proliferation and differentiation to single positive T–cells is promoted by the presence of IL–4 in the culture medium. The enriched population of IL–4 producing T–cells according to the invention show potential use as a source of cells for cell therapy in treating IL–4 response related disorders including certain autoimmune conditions such as diabetes mellitus.

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DEVELOPMENT OF REGULATORY CELLS AS A MEANS FOR TREATING AUTOIMMUNE DISEASE

FIELD OF THE INVENTION

This invention relates to processes for the production of selected T-cells. More specifically, the invention relates to processes for producing selected subpopulations of T-cells having desired cytokine profiles, and uses of such cells, for example in cell therapy.

BACKGROUND OF THE INVENTION

T-cells are a major form of lymphocytes and typically constitute a critical component of the mammalian immune system. In this application, the phrase "T-cells" includes both mature and immature T-cells. Immature T-cells (sometimes called thymocytes) are located in the thymus. While located in the thymus, immature T- cells are exposed to numerous growth factors secreted by thymic stromal cells. Immature T-cells also interact directly with thymic stromal cells during development, and such interaction is considered necessary to T-cell development in the thymus.

Immature T-cells undergo numerous changes while maturing in the thymus. Very immature T-cells lack detectable cluster of differentiation (CD) molecules 4 and 8, and are therefore described as $CD4^-$ $CD8^-$ (double negative) cells. During early T-cell development double negative (DN) cells undergo rearrangements in genes encoding cell surface molecules. Following this gene rearrangement, the majority of surviving T-cells begin expressing both CD4 and CD8 and are known as $CD4^+$ $CD8^+$ (double positive) cells. Double positive (DP) T-cells undergo a period of proliferation, which is followed by another genetic rearrangement to produce genes encoding a functional T-cell Receptor (TcR). Those developing T-cells which have undergone productive genetic rearrangements and survived to this point then enter the second major phase of development in the thymus, namely positive and negative selection.

While in the thymus, developing T-cells undergo selection which normally ensures that mature T-cells will recognize self MHC associated with foreign antigen. Thymic stromal cells, including thymic epithelial cells, are believe to play an important role in T-cell selection by providing high levels of self-MHC to which the developing T-cells may bind. The current understanding of this process is that during positive selection of T-cells in the thymus, the differentiation of DP cells into SP cells requires binding of the TcR and CD4 or CD8 co-receptors on DP thymocytes to antigenic peptide-MHC complexes on thymic epithelial cells. Those T-cells which do not bind self MHC, or which bind self antigens with high affinity ("self reactive T-cells"), generally undergo apoptosis and die. Developing T-cells which survive positive and negative selection in the thymus generally develop into either $CD4^+$ or $CD8^+$ single positive (SP) cells.

In their mature form, $CD4^+$ T-cells are MHC class II (MHC II) restricted, and $CD8^+$ T- cells are MHC class I (MHC I) restricted. The mechanism controlling the choice of a $CD4^+$ or a $CD8^+$ fate for developing T-cells is poorly understood. One current model suggests that TcR specificity for MHC class I or class II molecules may determine the lineage commitment of DP thymocytes into either $CD4^-CD8^+$ or $CD4^+CD8^-$ SP thymocytes by the down-regulation of CD4 or CD8 surface expression, respectively. Alternatively, a stochastic/selection model suggests that DP thymocytes may indiscriminately terminate synthesis of either CD4 or CD8, and that the subsequent maturation of DP thymocytes is dependent on the matched TcR and co-receptor specificity.

It is known in the art that treatment with phorbol 12-myristate, 13-acetate (PMA) plus ionomycin can stimulate DP thymocyte differentiation into $CD4^+CD8^-$ and/or $CD4^-CD8^+$ SP thymocytes without TcR engagement, and this differentiation is dependent on the duration and extent of stimulation. Phorbol esters such as PMA are believed to act by activating protein kinase C, an intermediate in the normal signal transduction pathway by bypassing the need for specific cell-surface receptor binding.

Two major functional types of T-cells are T-helper (Th) cells, and T-cytotoxic (Tc) cells. In general, Th cells are $CD4^+$ $CD8^-$ and Tc cells are $CD4^-$ $CD8^+$. However, these categorizations are not absolute and both $CD4^+$ Tc cells, and $CD8^+$ Th cells have been reported. Current understanding suggests that Tc cells are activated by binding to an antigen-MHC complex presented by an altered-self cell in the presence of IL-2. Activated Tc cells are effector cells which cause the lysis of cells presenting an antigen- MHC complex bound by the activated Tc cell.

Tc cells tend to secrete fewer cytokines than Th cells do. The most common cytokine secreted by Tc cells is IFNγ. However, a subtype of Tc cells, called Tc2 cells, has been reported to secrete IL-4.

Th cells are important to both humoral and cell-mediated immune responses. The current literature on Th cell activation suggests that Th cells are activated by the interaction of the TcR-CD3 complex on the Th cell surface with an antigen-MHC II complex on an antigen presenting cell (APC). This interaction is believed to trigger a series of events within the Th cell which result in cell proliferation, cell-surface receptor expression, and cytokine secretion. Activated Th cells may differentiate into memory cells or effector cells.

Th cells may belong to either the Th1 or Th2 subset of Th cells. The subsets Th1 and Th2 are best characterized by their cytokine production profiles. Th cells belonging to the Th1 subset ("Th1 cells") secrete interleukin (IL)-2, interferon (IFN)-γ, and tumour necrosis factor (TNF)-β. IL-2 may be necessary to T-cell activation. Th1 cells promote cell-mediated immune responses such as the induction of delayed- type hypersensitivity by way of macrophage activation, and the activation of cytotoxic T-cells. Th cells belonging to the Th2 subset ("Th2 cells") secrete IL-4, IL-5, IL-6, and IL-10. Th2 cells produce cytokines which favour a humoral immune response and function as effective helper cells in B-cell activation. Thus, the selective activation of Th1 or Th2 cell types may control the type of immune response which occurs.

In this application, the terms "Th2-like" and "Th2-type" cells refer to cells having a cytokine production profile similar to that typical of Th2 cells. However Th2 type cells and Th2-like cells as defined in this application need not necessarily be conventional Th2 cells. In this application, the terms "Th1-like" and "Th1-type" cells refer to cells having a cytokine production profile similar to that typical of Th1 cells. However, Th1-type cells and Th1-like cells as defined in this application need not necessarily be conventional Th1 cells.

The importance of the subtype of Th cell which is activated is increased by cross- regulation between Th1 and Th2 subtypes. The secretion of IFN-γ by Th1 cells inhibits the proliferation of Th2 cells, and the secretion of IL-10 by Th2 cells results in decreased activation and cytokine secretion by Th1 cells. Not only do the cytokines secreted by each Th cell subtype result in decreased activity by the other Th cell subtype, but in some cases the cytokines secreted by one Th cell subtype act to down regulate immune responses of the variety stimulated by the other Th cell subtype through pathways separate from the other Th cell type. For example, the secretion of IFNγ and IL-2 by Th1 cells inhibits the production of IgE which is stimulated by IL-4 produced by Th2 cells.

The manifestation and clinical outcome of some disorders appears to depend at least in part on the balance of cytokine production typically associated with Th1 and Th2-type immune responses. One widely studied disease in which the Th1/Th2 related cytokine response ratio is important is leprosy. Leprosy is caused by the infection of macrophages by the intracellular pathogen *Mycobacterium leprae*, which may trigger either a Th1 or a Th2-type immune response. In tuberculoid leprosy a Th1-type response occurs and the patient's body mounts a cell-mediated immune response which is usually effective in slowing disease progression and allowing the patient to survive. In contrast, in lepromatous leprosy a Th2-type response suppresses the cell-mediated immune response and a humoral response arises instead. This leads to a massive expansion of the pathogen load in the patient's tissues causing severe tissue damage and greatly diminished chances for survival.

Recent experiments have suggested that a shift from a Th1 dominant cytokine profile to a Th2 dominant cytokine profile correlates with disease progression in patients suffering from HIV infection. Th2 cells secrete IL-3, IL-4, IL-5 and IL-10.

Th1 : Th2 -like cytokine ratios also appear important to Type 1 hypersensitivity reactions. Type 1 hypersensitivity reactions include common allergic reactions such as hay fever and hives, as well as disorders such as asthma and systemic anaphylaxis. The mechanism for type 1 hypersensitivity reactions involves the antigen-induced crosslinking of IgE bound to mast cells and basophils, causing the release of vasoactive mediators. Cells having a Th1-like cytokine profile reduce type 1 hypersensitivity responses, whereas cells having a Th2-like cytokine profile tend to enhance them. The Th2-type secretions IL-3, IL-4, IL-5 and IL-10 affect B-cells, mast cells, and eosinophils to stimulate the reaction. In contrast, cells having a Th1-like cytokine profile secrete IFNγ which decreases IgE production, inhibits the proliferation of Th2 cells and inhibits the type 1 hypersensitivity response. Thus, the ratio of cells having Th1 and Th2-type cytokine profiles likely influences the balance of IFNγ and IL-4, and can potentially be used to control the onset and severity of type 1 hypersensitivity reactions.

There is evidence in the art indicating a role for the ratio of cells having Th1 and Th2 type cytokine profiles in the development and progression of autoimmune disease. The majority of organ-specific autoimmune diseases develop as a consequence of the activity of $CD4^+$ T-cells which react to self antigens. Experiments which have examined the Th1/Th2-type cytokine ratio in patients susceptible to or suffering from autoimmune disease have shown a tendency for high levels of Th1-type cells to promote autoimmune responses, whereas high levels of Th2-type cells tend to protect against disease development and slow the progression of manifested autoimmune disease.

Autoimmune diseases represent a major health problem in western countries in terms of both personal suffering and health care spending. Major T-cell mediated autoimmune disorders include insulin-dependent diabetes mellitus (IDDM),

and multiple sclerosis (MS).

IDDM is an organ-specific autoimmune disorder in which insulin-producing cells of the pancreas are destroyed as a result of the infiltration of the pancreas by large numbers of self-reactive T-cells which mediate delayed-type hypersensitivity. Experiments designed to correlate the relative propensity of individuals to mount Th1 or Th2 immune responses against glutamic acid decarboxylase, a known self antigen in IDDM, with IDDM susceptibility indicate that individuals prone to mount a Th1-type cell mediated immune response are more likely to develop IDDM and tend to show more rapid disease progression than those who mount a Th2-type humoral immune response.

An animal model for IDDM has been developed using a mouse strain called nonobese diabetic (NOD) mice. NOD mice spontaneously develop insulin dependant diabetes following the infiltration of the pancreas by large numbers of self-reactive T-cells.

A common feature of human IDDM patients and NOD mice is T-cell proliferative unresponsiveness. It has been proposed that this thymic T-cell anergy may lead to the breakdown of self-tolerance and the development of autoimmunity in NOD mice. Thymic T-cell anergy in NOD mice has been linked to defective signal transduction in response to TcR binding.

Thymic unresponsiveness in NOD mouse thymocytes has been reversed *in vitro* through the addition of exogenous IL-4 to the culture medium. *In vivo*, the administration of IL-4 to prediabetic NOD mice has been shown to protect them from the onset of diabetes during the treatment period. It has been shown that *in vitro*. NOD T-cells fail to product enough IL-4 to support their own proliferation, and exogenous IL-4 completely restored the proliferative capacity of these cells *in vitro*.

T-cells, as well as other cells of the immune system, express IL-4

receptors on their cell surface. Upon binding to its receptor, IL-4 is capable of increasing the responsiveness of that cell to IL-4 by signaling an increase in IL-4 receptor expression. IL-4 promotes the development of Th2 cells from naive T-cells upon antigen stimulation and can act to further expand and stimulate these Th cells. IL-4 also inhibits macrophage activation and can inhibit most of the macrophage activating effects of IFNγ.

Experiments using NOD mice have shown that T-cells from a diabetic NOD mouse are capable of causing diabetes by adoptive transfer into an otherwise normal mouse which has been irradiated to destroy its own immune system. Moreover, a NOD mouse which has not yet become diabetic and which has been irradiated to destroy its immune system can be protected from subsequent diabetes by the adoptive transfer of normal T-cells from a healthy donor. This is consistent with a model for insulin-dependant diabetes in which self-reactive T-cells are responsible for the onset of disease.

MS is a systemic autoimmune disease which affects the central nervous system. In MS, self-reactive T-cells cause inflammatory lesions on the myelin sheath of nerve fibers, causing the destruction of the myelin which insulates the nerve fibers. The resultant loss of myelin leads to serious neurological dysfunctions.

An experimental model for MS has been developed by injecting rats with myelin basic protein. This results in a disorder called experimental autoimmune encephalomyelitis (EAE) in which self-reactive T-cells infiltrate the myelin sheaths of the central nervous system, causing the destruction of the myelin and resulting in paralysis. When self- reactive T-cells are isolated from animals suffering from EAE and injected into healthy animals by adoptive transfer, the injected animals develop EAE. However, if the isolated T-cell population is divided into Th1 and Th2 cell subtypes, only Th1 cells will cause EAE by adoptive transfer. Th2 cells do not cause EAE by adoptive transfer and can also help to protect healthy mice from developing EAE if they are subsequently injected with myelin basic protein.

The different roles played by cells having Th1 and Th2-type cytokine profiles, and their ability to suppress the activities of the Th cell subtype with the alternate cytokine secretion profile, make it highly desirable to have a means of producing enriched populations of each cell type. However, the processes regulating the differentiation of T-cell subtypes is poorly understood. The leading model proposes that the differentiation of Th cells into Th1 or Th2 subtypes is determined by the cytokine environment at the time of Th cell interaction with antigen. In particular, the interaction of a Th cell with antigen in the presence of IL-4 is believed to be necessary for Th2 cell development, and the interaction of a Th cell with antigen in the presence of IL-12 is believed to be necessary for normal Th1 cell development.

The capacity to prevent or regulate certain disorders through the manipulation of the T-cell subtypes in the patient makes it desirable to have a process for the selective enrichment of particular T-cell subtypes.

Methods for the mechanical separation of Th1 and Th2 cells from mixed cell populations *in vitro* are known in the art. However, these methods require large initial cell populations in order to allow the recovery of useful quantities of Th1 or Th2 cells. Moreover, mechanical separation can prove stressful to cells and may reduce the usefulness of the recovered cells.

It is therefore desirable to have a process which will allow the production of large T-cell subpopulations with a desired cytokine profile from a range of starting materials without the need for mechanical separation. Additionally, thymic stromal cells which are normally present during T-cell development are a potential source of secreted factors which can complicate the regulation of culture conditions and which, if derived from a non-autologous source, may introduce pathogens into the culture. Thus, it is desirable to have a process which allows the use of a culture medium in which T-cells can proliferate and differentiate in the absence of thymic stromal cells.

SUMMARY OF THE INVENTION

It is thus an object of the present invention to provide a process for the in vitro expansion and proliferation of T-cells.

It is a further object of the present invention to provide a process for the proliferation and differentiation of immature T-cells (thymocytes) to single positive T-cells, with a preponderance of selected sub-populations of T-cells.

It is a further a more specific object of the present invention to provide a process for preparing enriched populations of Th2-like lymphocytes, useful in cell therapy to alleviate symptoms of an immunological disease.

The present invention utilizes a specific type of cell suspension conditioned culture medium CM, to promote the proliferation and differentiation of particular T-cell subtypes in the absence of thymic epithelial cells. As the specific examples demonstrate, primary murine thymocytes cultured in this CM-based medium expand about 5-20 fold during 4-6 days of culture, depending on the strain and age of the mice, and may be expanded still further by 10^2-10^3 fold upon serial passage of the thymocytes. In view of the very low concentration of IL-4 ($\leq$ 0.02 ng/ml) in CM, the use of CM allowed demonstration of the fact that IL-4 plays an important role in the regulation of thymocyte differentiation, and led to the finding that IL-4 augments the differentiation of $CD4^+CD8^-$ SP thymocytes and $CD4^-CD8^-$ $TcR\alpha\beta^+$ DN thymocytes, and that this differentiation correlates with the up-regulation of surface CD69 expression on $CD4^+CD8^+$ DP thymocytes. Thus, the disclosed process of culture in CM provides a means to selectively expand T-cell subtypes *in vitro*. From immature, double positive (DP) T-cells (thymocytes) CD4+CD8+, there can be selectively differentiated *in vitro* single positive lymphocytes, to provide a cell culture containing an enriched population thereof. Such cells can, in accordance with the present invention, be cultured *in vitro* to provide an enriched population of IL-4 producing cells useful in cell therapy to combat autoimmune disease such as diabetes mellitus.

Operating the process of the invention accordingly allows the production from a starting cell population exhibiting no IL-4 secreting cytokine profile, of a cell

population having significant, even predominant, Th2-type cytokine secretory profile, which includes IL-4 secretion, IL-3 secretion and IL-10 secretion. The process thus provides a means of obtaining cell populations useful in administration to patients having certain disorders, and a process of alleviating the symptoms of such disorders by administering to patients cell populations cultured according to processes described herein.

The present invention thus provides a mammalian T-cell population which is enriched in cells having a Th2-like cytokine production profile, e.g. IL-4 production, as compared with a similar T-cell population which has not been cultured using CM or XLCM™ as described in the culturing process herein. Such an enriched T-cell population provides cells for administration to patients to alleviate T-cell mediated disorders where Th1 : Th2 ratios are believed to be important, and disorders where IL-4 is effective. The enriched T-cell population of the invention may be produced by other methods besides culturing in the presence of CM or XLCM™ as described herein, for example by culturing in media containing only the essential ingredients of XLCM™ or ingredients functionally equivalent thereto, or by totally synthetic media. The T-cell population enriched in cells having a Th2-like cytokine production profile, useful as a source of cells for treating disorders as discussed above, constitute a preferred aspect of the present invention, independently of the method by which such populations are obtained. One skilled in the art can determine whether such an enriched cell population has been obtained by conducting simple routine experiments with his starting cell population and a CM described herein, and comparing the cytokine profile of the starting cell population with that resulting from culturing in the presence of CM as described.

The present invention thus provides a mammalian T-cell population which is capable of suppressing self-reactivity to specific self antigens by self-reactive T-cells, as compared with a similar T-cell population which has not been cultured using CM or XLCM™ as described in the culturing process herein. Such a self-reactivity suppressing T-cell population provides cells for administration to patients to alleviate

the symptoms of disorders where a T-cell mediated autoimmune response is believed to contribute to disease development, progression, or symptoms. The self-reactivity suppressing T-cell population of the invention may be produced by other methods besides culturing in the presence of CM or XLCM™ as described herein, for example by culturing in media containing only the essential ingredients of XLCM™ or ingredients functionally equivalent thereto, or by totally synthetic media. The self reactivity suppressing T-cell population, useful as a source of cells for treating certain disorders as discussed above, constitutes a preferred aspect of the present invention, independently of the method by which such populations are obtained. One skilled in the art can determine whether a self reactivity suppressing T-cell population has been obtained in respect of a particular disorder by conducting simple routine experiments with his starting cell population and a CM described herein, and comparing the impact of the introduction of a suitable quantity of cells from such a population into a suitable subject suffering from the autoimmune disorder with the impact of the introduction of a suitable quantity of cells from a population resulting from culturing in the presence of CM as described. For example, the generation of a population with self-reactivity suppressing properties in relation to autoimmune diabetes could be assayed by comparing the incidence of diabetes in a suitable Scid or otherwise MHC compatible subject which is disposed to develop autoimmune diabetes following the introduction of cells cultured in the presence of CM or XLCM™ according to the method of the invention with the effect of the introduction of the starting cell population.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other advantages of the invention will become apparent upon reading the following detailed description and upon referring to the figures in which:-

FIGURE 1 is a graphical presentation of the results of Example 1 below;

FIGURE 2(A-H) is a set of graphical and pictorial presentations of the results of Example 2 below;

FIGURES 3A and 3B are pictorial and graphical presentations of the results of Example 3 below;

FIGURES 4A and 4B are pictorial and graphical presentations of the results of Example 4 below;

FIGURE 4C is a pictorial presentation of the results of Example 4A below;

FIGURES 5A and 5B are pictorial and graphical presentations of the results of Example 5 below;

FIGURE 7 is (A) a pictorial presentation and (B) a graphical presentation of the results of Example 7 below;

FIGURE 8 is (A) a pictorial presentation and (B) is a graphical presentation of the results of Example 8 below;

FIGURE 9 is a graphical presentation of the results of Example 9 below;

FIGURE 10 is a graphical presentation of the results of Example 10 below;

FIGURE 11 is a graphical presentation of the results of Example 11 below; and

FIGURE 12 is a pictorial presentation of the results of Example 12;

FIGURE 13 is a graphical presentation of the results of Example 13 below;

FIGURE 14 is a graphical presentation of the results of Example 14

below;

FIGURE 15 is a graphical presentation of the results of Example 15 below;

FIGURE 16 is a graphical presentation of the results of Example 16 below;

FIGURE 17 is a graphical presentation of the results of Example 17 below;

FIGURE 18 is a graphical presentation of the results of Example 18 below;

FIGURES 19A and 19B are graphical presentations, and FIGURE 19C is a pictorial presentation, of the results of Example 19 below; and

FIGURE 20 A is a pictorial presentation of the procedure of Example 20, below.

FIGURE 20 B is a graphical presentation of the results of Example 20 below.

FIGURES 21A and 21 B are graphical presentations of the results of Example 21, below.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As embodied and broadly described herein, the present invention is directed to a process for the production of cell populations rich in T-cells which are capable of secreting significant quantities of IL-4, along with other cytokines

charactoristic of a Th2-like cell, to methods for culturing such subpopulations, to such subpopulations produced by this process, and to methods for the uses of these subpopulations in cell therapy and other appropriate medical applications. The culturing methods are in vitro methods using conditioned medium CM as described below, as a constituent of the cell culture medium. Whilst it is probable that the major proportion of the Th2-like cells produced herein are $CD4^+$ single positive cells, this is not necessarily or exclusively so. Certain of them may be $CD8^+$ single positive cells, or potentially even double positive of double negative cells. The common, defining charactoristic of the cell subpopulation enriched is their cytokine production profile which is charactoristic of Th2-like cells, especially in respect of the capacity to secrete IL-4.

According to one preferred embodiment of the invention, there is provided a process for the differentiation and proliferation of T-cells of various types, including DP thymocytes, i.e. CD4+CD8+ T-cells, to produce cell populations enriched in single positive T-cells, CD4+ or CD8+ and/or sub-populations of such cells, in a pre-selected manner, which comprises culturing the DP thymocytes in a special conditioned medium CM described herein, optionally also in the presence of added amounts of supplements such as cytokines and plasma.

Another, more specific preferred embodiment of the invention provides a method whereby a starting population of cells containing a complex mixture of T-cells, for example splenic cells, can be cultured *in vitro* to provide a population which is significantly enriched in a specific subset of T-cells, such as Th1-like or Th2-like cells. This provides a cell composition useful for introduction into the system of a mammalian patient for exerting protective effects against certain types of autoimmune disease. The starting cell population for use in the present invention is one which comprises T-cells. Accordingly, substantially any source of mammalian T-cells may be used as the starting cell population.

The conditioned medium CM used in the process of the present invention comprises a mixture of cell factors having a balance of stimulatory and inhibitory effects

favouring the proliferation of the desired cell population. The CM composition is produced by treating a cell population with an inducing agent which includes at least one plant mitogen. Preferred such plant mitogens include plant lectins such as concanavalin A (ConA) or phytohemagglutinin (PHA), and T-cell mitogens such as mezerein (Mzn) or tetradecanoyl phorbol acetate (TPA). Especially preferred is a combination of ConA and Mzn. Other mitogens of non-plant origin, including interferons of various kinds, may be used in addition. The starting cell population used to prepare the CM may comprise peripheral blood cells, umbilical cord blood cells, bone marrow cells, mixtures of two or more types of such cells, or fractions or mixed fractions of such types of cells. The starting cell population may be induced by adding the inducing agent(s) to an appropriate suspension thereof in aqueous, nutrient-containing medium. The CM inducing process may be affected by factors produced by the cells during culture, and by culturing conditions such as the medium used, temperature, time of culture, pH, exogenous recombinant growth factors, nutrients, etc. The medium used may be serum free.

A specific preferred example of a CM for use in the process of the present invention is XLCM™. The medium XLCM has been disclosed previously - see, for example, Skea et al., "Large ex vivo expansion and reduced alloreactivity of umbilical cord blood T- lymphocytes", Blood 90: 3680 (1997). It is further described in detail in the Materials and Methods, below. It may be derived from the supernatant of activated blood mononuclear cells. It can expand human cord blood T cells > 10^4-fold and adult T cells > 10^5-fold during a 4 week culture period. XLCM™ consists of many cytokines, some of which are present in high concentration (e.g. GM-CSF, IL-2 and IFN-γ) while others are present in extremely low amount (e.g. IL-4 and IL-7). In the processes of the invention, the CM as exemplified by XLCM is preferably used as an added ingredient to a standard cell culture medium, serum containing or serum free, in amounts in the approximate range of from 5% to 40% of the total volume of the culture medium.

XLCM™ supports the differentiation and proliferation of DP thymocytes

into single positive T-cells, as evidenced by experiments reported herein on cells from laboratory mice of various strains. When the culture medium additionally includes added amounts of exogenous IL-4, the differentiation of single positive CD4+ cells is strongly favoured, thereby providing a convenient and relatively rapid means for producing enhanced populations of CD4+, Th cells. Thus the process of the invention may also be used to promote the differentiation of DP into $CD4^+$ SP thymocytes, by culturing in XLCM™ in the absence of thymic stromal cells, when IL-4 is added to the culture. This differentiation is accompanied by an increase in CD69 surface expression on $CD4^+$ $CD8^+$ DP thymocytes, suggesting that IL-4 may provide and/or induce the signals required for the differentiation of DP thymocytes into $CD4^+$ SP thymocytes.

Moreover, culturing of splenic T-cells in the presence of XLCM has been found, in accordance with the present invention, to lead to differentiation of IL-4 producing cells, especially when conducted in the absence of splenic accessory cells (antigen presenting cells, APCs). Thus the present invention also provides a convenient and relatively rapid means for producing cell populations enriched in cells exhibiting a Th2-type cytokine production profile, or a cell population in which the ratio of Th1 cytokine profile cells : Th2 cytokine profile cells is shifted from the normal in favour of the Th2-like population. Such a shift influences the onset of certain autoimmune diseases, namely by delaying their onset. Accordingly such cell populations show potential in cell therapy for treating or delaying the development of certain autoimmune diseases such as IDDM.

As described in the specific examples below, this was demonstrated by culturing T-cells obtained from mouse strains by the process of the invention, and injecting the resultant cultured cell populations into mice of other strains, and then challenging the injected mice with autoimmune disease, namely IDDM. T-cells from the mouse strain nonobese diabetic (NOD) mice, which spontaneously develop IDDM, have been analyzed for their capacity to differentiate and proliferate in XLCM™. The profile of cytokine production from the resulting cell population evidences a shift in cell subpopulations in favour of cells having a Th2-like cytokine profile with a relative

decrease in Th1-like cells. Introduction of splenic cells from diabetic NOD mice, cultured in XLCM™ into NOD Scid mice protected these from the onset of IDDM. This provides further evidence of a shift in subpopulation in favour of cells having a Th2-like cytokine profile, and the utility of such enriched cell populations in conferring enhanced protection against the onset or development of autoimmune disease.

Upon administration of T cells cultured in XLCM to a subject genetically predisposed to autoimmune diabetes (i.e. a NOD mouse), the induction of diabetes by diabetogenic T-cells subsequently added, or the induction of diabetes by existing T-cells in non-diabetic NOD mice, is inhibited. The strength of the protective effect observed is related to the interval between the time of administration of the XLCM cultured cells and the time of challenge by diabetogenic cells. Moreover, T-cells removed from subjects genetically predisposed to diabetes in which disease progression has been delayed or prevented by the prior administration of XLCM-treated T-cells have a protective effect which inhibits the development of diabetes by a second subject receiving those cells. Thus, XLCM cultured T-cells introduced into a subject are able to exert a regulatory effect on subsequent pathogenic activity by T-cells removed from that subject.

When $CD4^+$and $CD8^+$ T-cell types are isolated prior to culturing in the presence of XLCM, followed by adoptive transfer into NOD.Scid mice, it becomes clear that culture in the presence of XLCM™ modulates the behaviour of both $CD4^+$ and $CD8^+$cells in a manner relevant to the regulation of autoimmune diabetes. In particular, $CD4^+$ T-cells which have been cultured in XLCM™ are less diabetogenic than uncultured cells, and the addition of IL-4 to the culture medium further reduces their diabetogenicity. $CD8^+$ T-cells cultured in XLCM do not appear to cause diabetes by adoptive transfer, and can act to protect against the development of diabetes resulting from challenge with uncultured diabetogenic cells.

The invention accordingly provides processes for generating cell populations for administration to patients to alleviate autoimmune diseases of the T-cell

mediated type, as exemplified by IDDM. Culturing of immature T-cells (thymocytes) in the presence of the medium described herein alters the cytokine secretion profile of cells within that population, generating an increased number of IL-4 producing cells. These IL-4 producing cells can be used as drug delivery means, and administered to patients to undergo *in vivo* secretion of cytokines such as IL-4 at locations where the IL-4 is most needed. This avoids many of the side effects of systemic cytokine administration.

In a patient suffering from IDDM, the defective immune system of the patient has effectively damaged the pancreatic islet cells so that adequate insulin quantities are no longer produced. Administration of fresh, effective islets to such patients e.g. by cell therapy in generally ineffective, since the immune system attacks the newly administered islet cells. The present invention provides a cell population which can be administered along with islet cells to a diabetic patient, to counteract the tendency of the patient's immune system to attack the islet cells, and without the need to provide special protection e.g. encapsulation of the islet cells.

The invention is exemplified and demonstrated in the following specific experimental examples.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Female NOD/Del (NOD) mice, MHC class I and class I deficient DKO NOD mice, NOD.Scid mice, and NOR mice were bred and maintained in our (University of Western Ontario, Robarts Institute, London, Ontario, Canada) specific pathogen free animal facility, and were used at 6-10 weeks of age.

Thymocyte isolation

Single cell thymocyte suspensions were prepared according to standard procedures. $CD4^+CD8^+$ DP thymocytes were purified by panning on anti-CD8 mAb coated culture dishes resulting in $\geq$ 95% $CD4^+CD8^+$ thymocytes and $\leq$ 1% $CD8^+$ SP thymocytes as assayed by two-color flow cytometry.

Medium, cytokines and antibodies

XLCM™ was prepared from human cord blood as described by Skea et al., op. cit. HBCM-2 medium consists of AIM-V serum-free medium (Life Technologies, Grand Island, NY) supplemented with 20 units/ml heparin (Organon Teknika, Inc, Toronto, ON), 50 μM 2-mercaptoethanol (2-ME, Life Technologies), 10 μg/ml gentamycin sulfate and 50 μg/ml streptomycin (Sigma, St. Louis, MO). RPMI-1640 (R5F) or DMEM (D10F) (both from Life Technologies) were supplemented with 5 or 10% fetal calf serum (FCS, Life Technologies), respectively, 2 mM glutamine, 50 μM 2-ME, 100 U/ml penicillin and 100 μg/ml streptomycin. EL4.IL-2 supernatant was prepared by stimulation of EL4.IL-2 thymoma cells (10^6/ml) (American Tissue Culture Collection (ATCC), Rockville, MD) in D10F, with 20 ng/ml PMA (Sigma) for 24 h. Recombinant IL-4 was purchased from R & D Systems (Minneapolis, MN). Rat anti-mouse CD8 (TIB- 210, clone 2.43, IgG2b) and rat anti-mouse IL-4 (HB-188, clone 11B11, IgG1) mAbs were generated from ATCC cell lines and purified from culture supernatants by protein G affinity chromatography.

Cell culture

Unfractionated or purified $CD4^+CD8^+$ DP thymocytes were cultured for 4–6 d at 37°C in HBCM-2 medium supplemented with 5% XLCM™ and with or without 2.5% human cord plasma (CP). CP was prepared according to standard procedures. Thymocytes were plated (2 x 10^5/well, 2 ml/well) in 24-well tissue culture plates (Nunc, Nalge Co. Rochester, NY). To monitor thymocyte expansion in XLCM™,

cells were harvested and subcultured at the indicated time points. Control cultures contained thymocytes maintained in either R5F supplemented with XLCM™ (5 or 10%) or EL4.IL-2 supernatant (25%). Where indicated, empirically determined optimal concentrations of IL-4 (5 ng/ml) or IL-4 plus the 11B11 anti-IL-4 mAb (10 μg/ml) were added to cultures.

Intracellular cytokine expression

Thymocytes cultured in XLCM™ were fluorescently stained for the cell surface markers CD3, CD4, and CD8 or the intracellular cytokines IL-2, IL-4, IL-10 and IFN-γ, respectively. For three-color surface marker staining, thymocytes were harvested on day 4-6 of culture depending on the cell density, washed in PBS/1% BSA/0.1% sodium azide, and were stained at 4°C for 30 min with PE-conjugated anti-mouse CD4 (clone: CT-CD4, rat IgG2a; Cedarlane, Hornby, ON, Canada), PE-Cy5-conjugated anti-mouse CD8a (clone: CT-CD8, rat IgG2a; Cedarlane) and FITC-conjugated anti-mouse CD3e (clone: 500-A2, hamster IgG; Cedarlane) or FITC-conjugated anti-mouse CD69 (clone: H1.2F3, Hamster IgG, PharMingen, San Diego, CA). For three-color intracellular cytokine staining, thymocytes were initially stained with FITC-conjugated anti-mouse CD4 (clone: RM4-5, rat IgG2a; PharMingen) and PE-Cy5-conjugated anti-mouse CD8a. After two washes, the cells were fixed with Cytofix/Cytoperm™ solution, followed by two washes with Perm/wash™ solution as instructed by the manufacturer (PharMingen). Cells were then stained for 30 min at 4°C with PE-conjugated rat anti- mouse mAbs to IL-2 (clone: JES6-5H4, IgG2b), IL-4 (clone: 11B11, IgG2b), IL-10 (clone: JES5-16E3, IgG2b) or IFN-γ (clone: XMG1.2, IgG1) (all mAbs supplied by PharMingen), respectively, washed twice with PBS and fixed in 0.5% paraformaldehyde in PBS prior to flow cytometric analysis. FITC-, PE-, and PE-Cy5- conjugated isotype mAbs were used as controls. Ten thousand events were collected on a FACScan cytometer and analyzed using CellQuest™ software (Becton Dickinson, Mountain View, CA).

Diabetes monitoring

Diabetes was monitored in mice by testing urine glucose twice a week. A mouse was classified as diabetic when it urine glucose level exceeded 56 mmol/L.

Analysis of XLCM

As shown in Table I, XLCM™ was determined by ELISA to contain various concentrations of several cytokines, including IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-12, IL-13, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). XLCM™ also contains the macrophage inflammatory protein-1α (MIP-1α) and MIP-1β C-C chemokines. IL-4 (≤ 0.02 ng/ml), IL-7 (≤ 0.001 ng/ml) and IL-15 (≤ 0.008 ng/ml) are present in relatively low concentrations, and do not increase relative to normal plasma (Table I). Some XLCM™ cytokines, e.g. IL-1β, TNF-α, TGF-β, and GM-CSF, may be derived from thymic stromal cells that play an important role in thymocyte differentiation and proliferation. This is expected as XLCM™ is derived from the supernatants of mitogen-activated human cord blood nucleated cells and cord blood is an enriched source of hematopoietic stem cells which may produce various cytokines upon activation. Thus, XLCM™ is enriched with thymic stromal cell derived factors and may influence thymocyte differentiation and proliferation.

Adoptive Transfer of Cells into NOD.Scid Mice

Splenic T-cells were isolated from a diabetic NOD mouseusing a T-cell Enrichment Column (R&D Systems) and cultured in HBCM-2 containing 5 % XLCM™ for 4 - 7 days under standard tissue culture conditions (initial plating density 200 000 cells per well in 24 well plates). The resultant cells (referred to as X-DT cells) were harvested and injected intraperitoneally (i.p.) (5 000 000 cells per mouse) into female NOD.Scid mice (6 - 8 weeks of age). In certain experiments, mice were also injected i.p. with uncultured diabetogenic cells from diabetic NOD mice (DT cells) (5 000 000 cells per mouse).

TABLE 1 - CYTOKINES AND CHEMOKINES IN XLCM

Cytokine	Concentration* in XLCM™ (ng/ml)	Range (ng/ml)	Increase Relative to Plasma**
IL-8	234	181-> 1000	5200
TNF-β	112	98-160	7000
MIP-1α	98	68-243	44
IL-2	44	12-159	244
TGF-β1	21	6.9-44	***
RANTES	15	4-54	1.5
MIP-1β	11	1-39	3.4
GM-CSF	11	0.7-24	11000
TNF-RII	9.1	6.8-17	1.1
IL-1β	6.4	0.2-18	2133
M-CSF	5.4	2.3-9.7	4.5
IL-13	3.6	1.5-13	300
IFN-γ	3.6	0.6-14	89
IL-1α	2.3	0.004-4.9	2300
IL-16	2.1	0.5-6	23
TNF-RI	1.8	1.1-2.4	***
Fas	1.3	<0.04-2.3	***
TNF-α	0.37	<0.001-3.4	370
IL-12	0.26	0.07-0.8	***
SCF	0.2	0.15-0.29	***
IL-10	0.02	0.007-0.2	5.7
IL-6	0.007	<0.006-0.028	***
IL-4	0.0068	0.00012-0.08	***
IL-7	<0.001	<0.001-0.024	***
IL-15	<0.008	<0.008	***

* median concentration of cytokine measured in n=6-18 independent lots of XLCM™ using commercial ELISA kits: IL-1β, IL-2, IL-4, IL-10, IL-12, RANTES, TNF-RI, TNF-RII, Fas (Cytoscreen™, Biosource International, Camarillo, CA), INF-γ (DuoSet™, Genzyme Diagnostics, Cambridge, MA), TNF-α, GM-CSF, MIP-1α (Cytokine Direct™, Intergen Company, Purchase, NY) and MIP-1β, TNF-β, IL-1α, SCF (Quantikineα™, R & D Systems, Minneapolis, MN)

** median concentration in XLCM/median concentration in plasma

*** not increased relative to plasma level

EXAMPLE 1 - XLCM supports thymocyte proliferation

The capacity of mouse thymocytes to proliferate in XLCM (5% XLCM in serum-free HBCM-2 medium) was determined. The results are shown in Figure 1, plots of numbers of cells per well against time of culture, in days. To obtain the upper plots *A*, C57BL/6 and NOD thymocytes were cultured in 5% XLCM in HBCM-2 medium in the presence or absence of 2.5% CP. To obtain the lower plots *B*, BALB/c thymocytes were cultured in 5% XLCM in HBCM-2 medium or R5F supplemented with either 5-10% XLCM or 25% EL4.IL-2 supernatant, harvested at day 4 and then serially passaged at the indicated times. All cultures were established at a cell density of 2 x 10^5/well in 2 ml in 24-well plates. The number of cells were counted in triplicate and expressed as the number of cells per well. The variation in numbers of cell/well between wells was less than 10% at each time point. Representative data from one of three reproducible experiments are shown.

Whereas NOD and BALB/c thymocytes plated at 2 x 10^5 cells/well expanded about 5-20 fold during culture for 4-6 days in XLCM, C57BL/6 thymocytes proliferated poorly (Fig. 1). Upon serial passage, thymocytes expanded by about 10^2- to 10^3-fold during 2-4 weeks in culture, and varied according to the strain (Fig. 1) and age of the mice (data not shown). NOD and C57BL/6 thymocyte proliferation was enhanced in the presence of added CP, especially during the initial 4 days of culture (Fig. 1A). IL-2 does not appear to be a major proliferative stimulus of thymocytes grown in XLCM, as BALB/c thymocytes proliferated poorly in R5F containing 25% EL4.IL-2 supernatant (Fig. 1B). Moreover, thymocytes proliferated more vigorously in HBCM-2 medium containing 5% XLCM than in R5F medium supplemented with 5-10% XLCM (Fig. 1B). These results indicate that XLCM is particularly supportive of thymocyte proliferation *in vitro*, and this level of proliferation is significantly enhanced by the addition of CP to the medium.

EXAMPLE 2 - XLCM promotes thymocyte differentiation

Proliferation of C57Bl/6 and NOD Thymocytes

The ability of thymocytes to differentiate during *in vitro* culture in XLCM was examined. C57BL/6 and NOD thymocytes (2×10^5/well) were cultured in 5% XLCM in HBCM-2 medium in the presence or absence of 2.5% CP, and were passaged at the indicated times shown on Fig 2A, which presents the results in graphical form, percentage of various cell types in the culture against time. At each time point, thymocytes were analyzed for the surface expression of CD3, CD4 and CD8 by three-color flow cytometry, and the percentages of $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$thymocytes were determined. Data shown are those for $CD3^{hi}$ thymocytes and represent one of three reproducible experiments.

In the presence of CP, more than 95% of C57BL/6 and NOD thymocytes were $CD3^+$ at days 4 and 7 of culture in XLCM(Fig. 2A). However, the patterns of differentiation in XLCM differed between C57BL/6 and NOD thymocytes, particularly after the first passage (day 4) of culture. C57BL/6 thymocytes consisted predominantly of $CD4^-CD8^+$ SP cells regardless of passage. In contrast, comparable numbers of $CD4^-CD8^+$ and $CD4^+CD8^-$NOD SP thymocytes were present in the initial cultures, whereas NOD $CD4^-CD8^+$ SP thymocytes were predominant after one passage (day 7). The proportion of C57BL/6 and NOD $CD4^+CD8^+$ DP thymocytes was reduced from 75-80% at day 0 to 15-20% at day 4 and 7 of culture in XLCM, suggesting that many DP thymocytes may have differentiated into SP thymocytes. Interestingly, the yield of NOD $CD4^+CD8^-$ SP thymocytes obtained in XLCM in the absence of CP (Fig. 2B) was higher than that in cultures supplemented with CP (Fig. 2A) and remained stable for several passages, suggesting that CP favors the differentiation of $CD4^-CD8^+$ SP thymocytes. Furthermore, in the absence of CP, the percentages of $CD4^+CD8^+$ DP and $CD4^-CD8^+$ SP thymocytes decreased in culture, while that of $CD4^-CD8^-$ thymocytes increased significantly (Fig. 2B). Despite the poor growth and low recovery of C57BL/6 thymocytes in the absence of CP, the thymocytes recovered consisted of a similar distribution of DP and SP subsets to that observed for C57BL/6 thymocytes cultured in the presence of CP (data not shown). Thus, XLCM promotes thymocyte differentiation, and the addition of CP preferentially stimulates the differentiation of $CD4^-CD8^+$ SP thymocytes.

Proliferation of MHC DKO NOD Thymocytes

In order to demonstrate that this effect did not derive from the proliferation of SP thymocytes possibly present in small numbers in the starting NOD and C57Bl/6 thymocyte populations, similar experiments were conducted using thymocytes from MHC class I and class II deficient DKO NOD mice. Such mice are devoid of SP thymocytes. This enabled analysis of the growth potential of DP thymocytes in the absence of any outgrowth of SP thymocytes. MHC DKO DP thymocytes were plated (3×10^6 cells/well in 2 ml of 5% XLCM™) in serum-free HBCM-2 medium, and CP was either used alone or was added to XLCM™ in control cultures. The number of viable cells recovered from the cultures of XLCM™, XLCM™ plus CP, CP or medium alone at various time points is shown in Figure 2C. During the first day of culture, the number of cells decreased about 95% in XLCM™, 75% in XLCM plus CP, 55% in CP and 65% in medium alone. The number of cells recovered in the cultures of CP or medium alone progressively declined over time and no viable cells were recovered on days 3 and 5, respectively. In contrast, the number of cells recovered in XLCM™ or XLCM™ plus CP remained the same on day 2, and actually increased progressively during the next 3 days of culture. The number of cells recovered in XLCM™ on day 5 was increased about 10-fold compared to that recovered on day 1. The DP thymocyte proliferative response obtained in XLCM™ plus CP paralleled that of XLCM™, but the numbers of viable cells recovered in XLCM™ plus CP cultures exceeded that detected in XLCM™ cultures at all time points (Fig. 2C).

Analyses of the cell size showed that >80% of the cells recovered in XLCM™ after 1 day of culture were large blasts as determined by flow cytometry (FSC) (Fig. 2F). At this point, however, both small and large cells that formed cell aggregates were observed in XLCM™ plus CP cultures, with the percentage (14.3%) of large cells being less than that of small cells. Note that the percentage of large blasts obtained in XLCM™ plus CP was also considerably less than that obtained in XLCM™ after day 1 (Fig. 2F) but on day 3 of the culture the cell recoveries from XLCM™ and XLCM™ plus CP were comparable (Fig. 2C) inset). Interestingly, the switch in yield from small

to large cells in XLCM™ plus CP occurred on day 4 of culture, as small cells were not observed at this time (data not shown). Predominantly small cells were recovered from cultures in CP or medium alone throughout the culture period, and these cells were essentially dead by day 5 of culture (Fig. 2C).

Cell cycle analyses consistently showed that during the first day of culture, cells in S-phase of the cell cycle comprised about 30% in XLCM and about 8% in XLCM™ plus CP (Fig. 2D). The percentages of cells in S-phase increased over time and reached a plateau between days 2 and 3 of culture in XLCM™ or XLCM™ plus CP (Fig. 2D). In contrast, cells cultured in CP or medium alone did not enter S-phase of the cell cycle (data not shown).

To further determine the potency of XLCM™ in potentiating thymocyte growth, the dose-dependency of XLCM™ on thymocyte proliferation was examined. Fig. 2E shows that MHC DKO thymocyte proliferation as measured by the uptake of tritiated thymidine in XLCM™ is dose-dependent. The presence of CP alone in culture did not promote thymocyte proliferation, but CP significantly enhanced thymocyte proliferation when added to XLCM™ . Taken together, these findings indicate that XLCM™ can selectively induce both the death and growth of DP thymocytes. CP alone has no effect on thymocyte proliferation, but CP partially inhibits cell death and moreover enhances thymocyte proliferation when present in XLCM™ .

Differentiation of MHC DKO NOD Thymocytes

The ability of DP thymocytes from MHC DKO NOD mice to differentiate into SP cells during *in vitro* culture in XLCM™ was examined. Since DKO mice are devoid of SP thymocytes analyses of DKO DP thymocytes enabled investigation of whether SP thymocytes that grow in XLCM™ arise from the differentiation of DP thymocytes rather than from the selective outgrowth of SP T-cells that may contaminate a population of wild-type NOD DP thymocytes.

MHC DKO DP thymocytes were cultured in 5% XLCM™ , harvested at various times and the surface antigen phenotype of viable cells was analyzed by flow cytometry. XLCM™ stimulated $CD4^+CD8^+$ DP thymocytes to differentiate into $CD4^+CD8^-$ (8.3%) and $CD4^-CD8^+$ (12.3%) SP thymocytes as well as $CD4^-CD8^-$ DN (41.8%) thymocytes during the first day of culture (Fig. 2G). The percentage of DP thymocytes recovered at this time was significantly reduced from 98% (before culture) to 38%. After 3 and 5 days of culture, the percentages of DP thymocytes were further decreased and this was accompanied by increased percentages of SP and DN thymocytes (Fig. 2H). This differentiation of DP to SP and DN thymocytes was delayed in XLCM™ + CP, (Figs. 2G and 2H). This is consistent with our findings that the numbers of cells in S-phase (Fig. 2D) and large cells (Fig. 2F) were low in XLCM™ + CP at this time. Subsequently, the percentage of DP thymocytes was reduced, and that of SP and DN cells increased slowly during a 3 day culture. Interestingly, the percentage of DP cells was diminished dramatically (<10%) on day 4, and this was accompanied by a switch of small to large cells, indicative of the growth and differentiation of DP thymocytes in XLCM™ + CP. The phenotypes of thymocytes on day 4 (data not shown) were similar to that on day 5 (Fig. 2H). Interestingly, DP thymocytes differentiated mainly into $CD4^-CD8^+$ SP and $CD4^-CD8^-$ DN cells in XLCM™ and XLCM™ + CP (Fig. 2H).

Example 3 - XLCM™ mediated DP thymocyte differentiation is associated with the upregulated surface expression TCR, CD25, CD44, CD69 and CD40L

During the development of $CD4^+CD8^+$+ DP thymocytes into $CD4^+$ or $CD8^+$ SP thymocytes, the surface expression of TCR as well other markers of T cell maturation (e.g. CD69, CD44, and CD25) may be upregulated. To determine whether thymocytes cultured in XLCM™ or XLCM™ plus CP are phenotypically mature, their surface expression of TCR, CD25, CD69, CD44 and CD40L was examined. MHC DKO thymocytes were cultured in XLCM™, harvested at various times, stained with fluorochrome-conjugated mAbs to CD4, CD8 and TCRαβ and analyzed by flow cytometry. Expression of TCRαβ on $CD4^+CD8^-$ and $CD4^-CD8^+$ SP as well as

$CD4^+CD8^+$ DP thymocytes was upregulated during the first 3 days of culture in XLCM™ (Fig. 3A). In contrast, $CD4^-CD8^-$ DN thymocytes were either $TCR\alpha\beta^{low}$ or $TCR\gamma\delta$ (data not shown), indicating that some MHC DKO thymocytes cultured in XLCM™ were able to differentiate into $TCR\gamma\delta$ thymocytes. Similar results were obtained for thymocytes cultured in XLCM™ + CP.

Before culture, MHC DKO thymocytes were $CD25^-$, $CD44^{intermediate}$ and $CD69^-$. Culture of these thymocytes in XLCM™ or XLCM™ plus CP for 5 days resulted in the elevated surface expression of CD25, CD44 and CD69, as reflected by increased percentages of $CD25^+$, $CD44^{high}$ and $CD69^+$ thymocytes (Fig. 3B). Whereas the expression of these surface markers was upregulated on all thymocyte subsets, CD69 expression was enhanced predominantly on $CD4^+CD8^+$ DP cells. CD25 expression was increased on $CD4^+CD8^-$ and $CD4^-CD8^+$ SP cells (data not shown). Culture of thymocytes in XLCM™ or XLCM™ plus CP resulted in the increased surface expression of CD40L on about 1% of these thymocytes, suggesting that the latter thymocytes acquired a mature and activated T cell phenotype (Fig. 3B).

Example 4 - Thymocytes differentiated in XLCM™ produce cytokines and respond to syngeneic and allogeneic MHC alloantigens

To examine whether MHC DKO thymocytes differentiated in XLCM™ are functionally mature, the capacity to produce Th1 and Th2 like cytokines and to respond to MHC alloantigens was determined. IL-2, IL-4, IL-10 and IFN-γ producing thymocytes were enumerated by intracellular cytokine fluorescent staining. About 5-10% of MHC DKO thymocytes cultured in XLCM™ for 5 days secreted IL-2, IL-4, IL-10 and IFN-γ (Fig. 4A). The percentage of IL-4 or IL-10 producing cells was slightly higher than that of IFN-γ-producing cells. Both $CD4^+CD8^-$ and $CD4^-CD8^+$cytokine-producing SP thymocytes were detected (data not shown). It is unlikely that these cytokine-producing cells resulted from the outgrowth of pre-existing mature SP thymocytes, as MHC DKO mice are devoid of SP cells and no cytokine-producing cells were detected in freshly isolated thymocytes.

In addition to their capacity to produce cytokines, MHC DKO thymocytes that differentiated in XLCM™ responded to syngeneic NOD ($H\text{-}2^{g7}$) and allogeneic (BALB/c, $H\text{-}2^{d}$; and C57BL/6, $H\text{-}2^{b}$) MHC antigens present on irradiated stimulator splenocytes (Fig. 4B). The response of these MHC DKO thymocytes to $H\text{-}2^{b}$ alloantigens was lower than that to $H\text{-}2^{d}$ alloantigens, which may be explained in part by the deficiency of I-E molecules expressed by antigen presenting cells (APCs) from C57BL/6 ($H\text{-}2^{b}$) mice. These results indicate that thymocytes that differentiate in XLCM™ are functionally mature.

EXAMPLE 5 - IL-4 regulates DP thymocyte differentiation in XLCM in the absence of thymic epithelial cells

C57BL/6 and NOD $CD4^{+}CD8^{+}$ DP thymocytes were isolated by panning on anti-CD8 mAb coated dishes and shown by flow cytometry to be $\geq$ 95% $CD4^{+}CD8^{+}$ DP cells. Thus, the purified population of DP cells was largely devoid of thymic stromal cells. The purified DP cells were cultured (2×10^{5}/well) in 5% XLCM in HBCM-2 medium in the presence or absence of CP (2.5%) or CP plus IL-4 (5 ng/ml). Thymocytes were harvested on days 5-6 and were stained with PE-conjugated anti-mouse CD4 and PE-Cy5-conjugated anti-mouse CD8 mAbs. Numbers in quadrants represent percentages of positive cells, and the data shown are from one of two representative experiments with similar results.

C57BL/6 and NOD DP thymocytes not only proliferated well but also differentiated under these conditions (Fig. 5A). The patterns of differentiation of C57BL/6 and NOD DP thymocytes were similar to those observed for unfractionated thymocyte populations (Figure 2A), as C57BL/6 $CD4^{-}CD8^{+}$ and NOD $CD4^{+}CD8^{-}$ SP thymocytes, respectively, were found to be predominant.

The addition of exogenous IL-4 to cultures promoted the differentiation of $CD4^{+}CD8^{-}$ SP and $CD4^{-}CD8^{-}$ DN thymocytes and diminished the proportion of

$CD4^-CD8^+$ SP and $CD4^+CD8^+$ DP thymocytes. The DN thymocytes induced to differentiate by IL-4 express TcRαβ but not TcRγδ. These results indicate that DP thymocytes can differentiate into SP thymocytes in XLCM in the absence of thymic stromal cells, and that IL-4 can promote thymocyte differentiation independently of thymic stromal cells.

IL-4 also influenced the differentiation of MHC DKO DP thymocytes in the absence of thymic stromal cells. Purified MHC DKO DP thymocytes were cultured for 5 days in XLCM™ in the presence or absence of CP and/or IL-4, and then analyzed for their surface expression of CD4 and CD8. In the presence of CP, addition of IL-4 (5 ng/ml) to culture decreased the percentage of $CD4^-CD8^-$ DN (from 37.9% to 16.1%) thymocytes, and markedly increased the percentages of $CD4^+CD8^+$ DP (from 8.4% to 18.5%) and $CD4^+CD8^-$ SP (from 5% to 20.7%) thymocytes. Similar results were observed when CP was absent from the cultures. As shown in Figure 5B, the effect of Il-4 on thymocyte differentiation was completely blocked in the presence of an anti-IL-4 mAb. Other cytokines such as IL-7, IL-10, IL-12 and IL-15 did not significantly alter $CD4^-CD8^-$ DKO DP thymocyte differentiation when added to XLCM™ based culture. Thymocyte proliferation in XLCM™ was enhanced by IL-4 but not IL-7, IL-10, IL-12 or IL-15, as depicted in Figure 5C).

EXAMPLE 6 Il-4 regulates thymocyte proliferation and differentiation in XLCM™

Since XLCM™ promotes both thymocyte proliferation and differentiation, this culture medium was used to further analyze the role of a given cytokine(s) in thymocyte differentiation. XLCM™ contains very low amounts of IL-4, IL-7, IL-10, IL-12 and IL-15, which do not exceed the levels relative to normal plasma, with the exception of IL-10 (Table 1). IL-4 is involved in the regulation of thymocyte development, but its precise role is not fully understood. The question of whether exogenous IL-4 influences thymocyte differentiation in this XLCM™-based culture system was investigated.

C57BL/6 and NOD thymocytes (2 x 10^5/well) were cultured in 5% XLCM in HBCM-2 medium in the presence of 2.5% CP. IL-4 (5 ng/ml) or IL-4 plus anti-IL-4 mAb (10 mg/ml) was added to cultures. Thymocytes were harvested on day 6, stained with PE-conjugated anti-mouse CD4 and PE-Cy5-conjugated anti-CD8 mAbs, and analyzed by flow cytometry. The results are shown in Fig. 6A. The numbers in quadrants represent percentages of positive cells. Representative data from one of three reproducible experiments are shown.

In the presence of 2.5% CP, addition of IL-4 (5 ng/ml) to cultures markedly increased the frequencies of $CD4^+CD8^-$ SP and $CD4^-CD8^-$ DN thymocytes but decreased the frequencies of $CD4^-CD8^+$ SP and $CD4^+CD8^+$ DP thymocytes from both mouse strains (Fig. 6). Similar results were obtained when CP was absent from cultures. These patterns of IL-4 induced thymocyte differentiation were completely blocked by addition of anti-IL-4 mAb to cultures. Thus, IL-4 induces mouse thymocyte differentiation in XLCM in a strain-independent manner.

EXAMPLE 7 - IL-4-mediated thymocyte differentiation is associated with an increase of surface CD69 expression

Since expression of CD69 on thymocytes may correlate with negative and positive thymocyte selection, experiments were conducted to investigate whether a change in CD69 surface expression is associated with IL-4-induced thymocyte differentiation. C57BL/6 and NOD $CD4^+CD8^+$ DP thymocytes isolated by panning on anti-CD8 mAb coated dishes and shown by flow cytometry to be $\geq$ 95% $CD4^+CD8^+$ DP cells were cultured (2 x 10^5/well) in 5% XLCM in HBCM-2 medium in the presence or absence of CP (2.5%) or CP plus IL-4 (5 ng/ml). Thymocytes were harvested on days 5-6 and were stained with PE-conjugated anti-mouse CD4, PE-Cy5-conjugated anti-mouse CD8 and FITC conjugated anti CD69 mAbs.

Figure 7A shows that C57BL/6 thymocytes express a basal level of surface CD69 when cultured in XLCM in the absence of exogenous IL-4. Exposure to

IL-4 elevated the surface expression of CD69 on thymocytes as reflected by an increase in both the percentage of $CD69^+$ thymocytes and the mean fluorescence intensity (MFI). Although upregulation of CD69 expression by IL-4 was observed on $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ thymocytes, the most significant increase (~25%) in CD69 expression was observed on $CD4^+CD8^+$ DP thymocytes (Fig. 7A). The effect of IL-4 on CD69 expression was almost completely blocked when anti-IL-4 mAb was added to cultures (Fig. 7A). IL-4 treatment increased the MFI values for CD69 expression on NOD and NOR thymocytes, although the percentages of $CD69^+$ cells in these thymocyte populations did not increase significantly (Fig. 7B).

EXAMPLE 8 - XLCM-induced Th2-like cytokine production by thymocytes is enhanced by IL-4

To determine the T-cell subtypes produced by culture in XLCM, the cytokine secretion profiles of thymocytes cultured in XLCM were analyzed. The ability of exogenous IL-4 or cord plasma (CP) to enhance the expansion of particular T-cell subtypes was examined. IL-2, IL-4, IL-10 and IFN-γ producing thymocytes were enumerated by three-color fluorescent intracellular cytokine staining in conjunction with cell surface staining for $CD4^+$ and $CD8^+$ thymocyte subsets, as shown in Fig. 8A. C57BL/6 and NOD thymocytes were cultured (2×10^5/well) in 5% XLCM in HBCM-2 for 4-6 days. CP (2.5%) and/or IL-4 (5 ng/ml) were added to cultures, as indicated. Thymocytes were harvested and stained with FITC-conjugated anti-CD4 and PE-Cy5-conjugated anti-CD8 mAbs, and then intracellularly stained with PE-conjugated anti-mouse IL-2, IL-4, IL-10 or IFN-γ mAbs, respectively. Data were analyzed by three-color flow cytometry, and results are expressed as percentages of cytokine-producing cells. Data shown are from one of three representative experiments with similar results. *A*, Fluorescence profiles of intracellular expression of IL-4 by $CD4^+$ and $CD8^+$ NOD thymocytes. *B*, Histograms of intracellular cytokine expression by ungated thymocytes and gated $CD4^+CD8^-$ and $CD4^-CD8^+$ SP thymocytes.

Although C57BL/6 thymocytes proliferate weakly in the absence of CP, sufficient cells for flow cytometric analysis were obtained. C57BL/6 and NOD

thymocytes cultured in XLCM were found to secrete IL-2, IL-4, IL-10 and IFN-γ, but mainly IL-4 and IL-10 (Fig. 8B). The frequencies of IL-4 and IL-10-producing NOD thymocytes were higher than those of C57BL/6 thymocytes. Addition of exogenous IL-4 to XLCM increased the frequencies of IL-4 and IL-10-producing C57BL/6 but not NOD unfractionated thymocytes (Fig. 8, A and B). In contrast, addition of CP to cultures strongly inhibited cytokine production by both C57BL/6 and NOD thymocytes. This inhibition was partially reversed by addition of exogenous IL-4, which increased the frequencies of IL-4- and especially IL-10- producing thymocytes (Fig. 8B). These results indicate that XLCM preferentially supports the differentiation of thymocytes that secrete Th2-type cytokines, and the frequencies of the latter thymocytes can be enhanced in culture by the addition of IL-4 and inhibited by the addition of CP.

EXAMPLE 9 XLCM Stimulates Splenic T-cell Proliferation

The experiments reported in Examples 2 and 5 considered the effect of culture in XLCM on thymocytes. However, thymocytes represent an unusual T-cell population because of the high percentage of immature T-cells as compared to other regions of the body. As clinical treatments generally rely on more easily accessible sources of T-cells, such as peripheral blood, it was desirable to assess the effect of XLCM culture on a more mature T-cell population. Peripheral blood is not a practical source of T-cells from mice. Instead, secondary lymphoid organs, and in particular the spleen, was selected as a source of predominantly mature T-cells for the examination of cell proliferation and phenotype following culture in XLCM.

Splenocytes from C57Bl/6 and NOD mice were cultured in HBCM-2 medium containing 5 % XLCM with or without cord plasma (CP) and survival was assessed after 4, 7, and 11 days in culture. The results of this experiment are depicted in Figure 9A.

CP enhanced the survival of C57Bl/6 T-cells during the first 7 days of culture, but survival at 11 days was higher in the group which were cultured in the absence of CP. NOD mouse T-cells cultured in the presence of CP had a somewhat lower survival rate than those cultured without CP until day 11 of culture at which point

most of the cells in both groups had died.

Splenocytes from BALB/c mice were cultured in HBCM-2 medium containing 5 % XLCM or R5F medium with or without 5 % or 10 % XLCM at a plating density of 200 000 cells / well in 24-well plates. The cells were harvested, counted, and passaged (at 200 000 cells / well) at days 4, 7, 11, 18 and 24. (Figure 9 B)

BALB/c T-cells cultured in the absence of XLCM died within the first 7 days of culture, with fewer than 1 000 000 cells /1 well surviving to passage at day 4. In contrast, T-cells cultured in R5F with the addition of 5 % XLCM had in excess of 3 000 000 survival as of day 4 of culture and had some viable cells in culture as of day 11. T-cells cultured in the presence of 10 % XLCM had approximately 2 500 000 surviving cells / well as of day 4 of culture, and also had some viable cells as of day 11 of culture. T-cells cultured in HBCM-2 plus 5 % XLCM had over 3 500 000 surviving cells /well on day 4. This population declined to just over 1 000 000 cells / well on day 11 and began increasing after day 18, reaching a population in excess of 2 500 000 cells / well on day 24.

Thus, XLCM can prolong the survival of T-cells from spleen *in vitro*.

EXAMPLE 10 PHENOTYPES OF SPLENOCYTES EXPANDED IN XLCM

Splenocytes from C57Bl/6 and NOD mice were cultured in HBCM-2 medium containing 5 % XLCM in the absence (Figure 10 A) or presence (Figure 10 B) of CP in 24-well plates. The cells were harvested and sequentially passaged, and their phenotype was determined using flow cytometry. T-cells cultured in XLCM in the absence of CP are predominately single positive cells.

In splenocytes from C57Bl/6 mice, the addition of cord plasma appears to disfavour the expression of $CD4^+$, and favour the expression of $CD8^+$ early in culture. The addition of CP correlated with a decrease in $CD4^-$ $CD8^-$ DN cells in later culture.

Substantially the same impact of CP was observed in splenocytes from NOD mice. However, in splenocytes from NOD mice there was also a much more pronounced impact of CP on the percentage of $CD3^+$ cells than there was with C57Bl/6 splenocytes. In splenocytes from NOD mice, the addition of CP caused an increase in $CD3^+$ cells from approximately 60 % (no CP) to in excess of 80 % in early culture and from approximately 40 % to approximately 80 % later in culture.

EXAMPLE 11 IL-4 ENHANCES THE DIFFERENTIATION OF XLCM-INDUCED IL-4 PRODUCING CELLS

Example 5 indicated that IL-4 could enhance thymocyte differentiation in XLCM. It was therefore possible that IL-4 might have some effect on spleen T cells cultured in XLCM. However, as spleen T-cells are predominantly SP cells, it was unclear what effect, if any, IL-4 would have.

Splenic T-cells from C57Bl/6 or NOD mice were cultured in HBCM-2 medium containing 5 % XLCM in the presence or absence of IL-4 (5 ng/ml) and/or CP for 5-6 days at which time they were harvested and their cytokine production profiles were measured. Figure 11 depicts the result obtained for (A) total T-cells, (B) $CD4^+$ cells, and (C) $CD8^+$ cells.

Cultures of total C57Bl/6-derived splenic T-cells exposed to XLCM with no further additions contained more IL-4 and IL-10 producing cells than IFNγ and IL-2 producing cells; however, IL-4 producing cells formed only approximately 10 % of the cell population. However, when exogenous IL-4 was added to the culture medium, IL-4 producing cells increased to nearly 20 % of the cell population. The addition of CP in conjunction with IL-4 reduced the overall percentage of IL-4 producing cells in culture to lower levels than those observed with XLCM alone. The addition of CP without IL-4 reduced the production of all measured cytokines to levels below those observed with XLCM alone. When C57Bl/6 splenic T-cell cytokine production was examined for $CD4^+$ and $CD8^+$ cells separately, a similar effect of IL-4 production was

observed. However, it also became apparent that the addition of IL-4 depresses the production of IL-2 by both $CD4^+$ and $CD8^+$ cells.

In parallel to C57Bl/6 cultures, cultures of total NOD-derived splenic T-cells exposed to XLCM with no further additions contained more IL-4 and IL-10 producing cells than IFNγ and IL-2 producing cells; however, as with the C57Bl/6 culture, IL-4 producing cells formed only a small proportion of the total cell population. IL-4 producing cells comprised only approximately 5 % of the total T-cell population. The addition of exogenous IL-4 to the culture medium resulted in a moderate increase in the percent of IL-4 producing cells, however, these cells still represented less than 10 % of the total T-cell population. In contrast to the results observed in respect of C57Bl/6 cells, the addition of both IL-4 and CP to NOD T-cell culture increased the percentage of IL-4 producing cells above the level observed for IL-4 only. The addition of IL-4 to cultures of NOD T-cells did not significantly increase the percentage of cells in the population producing IFNγ.

Thus, IL-4 enhances the differentiation of XLCM-induced IL-4 producing cells.

EXAMPLE 12 EFFECT OF XLCM ON DIABETOGENIC T-CELL DIFFERENTIATION: PROMOTION OF IL-4 PRODUCING CELL DIFFERENTIATION

Example 8 indicated that culture in XLCM caused enrichment of Th2-type cells from a starting population of thymocytes. As the Th2 : Th1 ratio may influence the behaviour of self-reactive cells and may thereby be important to the regulation of certain autoimmune diseases, including IDDM, it would be useful to have a means of predicably altering the Th1 : Th2 ratio in T-cell populations drawn from tissues other than thymus. In order to investigate the effect of XLCM culture on a more mature T-cell population than that found in thymus, the effect of XLCM culture on T-cells from spleen was examined. The NOD mouse strain was selected because a

suboptimal Th2 cell level in NOD T-cell populations has been postulated to play a role in the development of diabetes in these animals. It would, therefore, be useful to know if the predictable adjustment of the Th2 : Th1 ratio in such cell populations was possible.

A T-cell population containing self-reactive T-cells was purified from the spleens of NOD mice with active diabetes using a T Cell Enrichment Column (R&D Systems) and cultured at a plating density of 200 000 cells / well in 24-well plates. In NOD mice, self reactive T-cells are diabetogenic, because they mediate an immune response to self antigens on pancreatic cells, leading to cell loss and a corresponding loss of insulin-producing capability. If left untreated, NOD mice typically develop autoimmune diabetes at an early age. The NOD T-cells were cultured in either HBCM-2 medium only in antiCD3 coated plates, or in HBCM-2 medium containing 5 % XLCM. After 48 hours the cells were harvested and the cytokine profiles of $CD4^+$ cells was assessed using flow cytometry. The results of this experiment are depicted in Figure 12. Cells cultured in XLCM express the Th2-type cytokines IL-4 and IL-10 more highly than cells grown in HBCM-2 alone in the presence of anti CD3. The level of IFNγ, a Th1-type cytokine, was not increased by culture in XLCM. Thus, culture of NOD splenocytes in XLCM selectively enhances the expression of cytokines typically associated with the Th2 phenotype.

EXAMPLE 13 T-CELLS ISOLATED FROM DIABETIC MICE AND CULTURED IN XLCM™ ARE NOT PATHOGENIC AND DELAY ADOPTIVE TRANSFER OF IDDM

In light of Example 12 and the cross-regulation of Th2 and Th1-type cytokine related activities, the question whether NOD Th2-like cells produced by the methods of Example 11 could influence the development of diabetes in pre-diabetic NOD mice was investigated.

A population of T-cells containing self-reactive T-cells was isolated from the spleens of NOD mice with active diabetes and cultured in XLCM for 6 days. The cultured cells (X-

DT) were adoptively transferred (5 000 000 cells per mouse) into NOD.SCID mice either alone or in conjunction with uncultured diabetogenic cells (DT) (5 000 000). Control mice received only DT cells (5 000 000 cells per mouse). The results of this experiment are depicted in Figure 13.

Diabetes was apparent in some control animals by 14 days following transfer, and all control animals were diabetic within 30 days of transfer. In contrast, animals which received X-DT cells remained diabetes-free for the full 58 day observation period. Those animals which received X-DT and DT cells together developed diabetes within the observation period; however, the time of onset was later than was observed in the animals which received only DT cells. The time of earliest onset was delayed by approximately10 days, and the time of latest onset was delayed by approximately 20 days, providing an average 15 day delay in the time of onset. This indicates that not only does culture in XLCM inhibit the diabetogenic characteristics of otherwise diabetogenic self-reactive cells, but these cultured cells can also undertake a protective function *in vivo* when transferred into an animal challenged by diabetogenic cells.

EXAMPLE 14 KINETICS OF PROTECTION FROM IDDM CONFERRED BY ADOPTIVE TRANSFER OF X-DT CELLS TO NOD.SCID RECIPIENT MICE

In Example 13, X-DT cells were transferred at the same time as uncultured DT cells. To investigate whether the regulatory effect of the transferred Th2 cells would persist once those cells were removed from XLCM and placed in the environment of the host animal, the effect of a delay between the transfer of X-DT cells into an animal and the challenge of that animal with DT cells was examined.

T-cells were isolated from NOD mice expressing active diabetes. These cells were cultured in XLCM for 6 days, at which point they (X-DT) were harvested and injected (5 000 000 cells / mouse) into NOD.Scid mice. Mice in which diabetes was

not detected were challenged by the further injection 5 000 000 uncultured T-cells from NOD mice expressing active diabetes (DT). Mice were challenged with DT cells at either 1 week, 6 weeks, or 13 weeks following initial injection with X-DT cells, and the incidence of detectable diabetes in these animals was monitored. The results of this experiment are depicted in Figure 14.

By week 3, diabetes was detectable in some mice of the challenged with DT cells in week 1. All these mice suffered from detectable diabetes by week 4. In contrast, animals which were not challenged with DT cells until week 6 did not suffer from detectable diabetes until week 9, and some of these mice did not suffer from detectable diabetes until week 17, fully 11 weeks after DT challenge. Thus, the protective effect of X-DT cells is enhanced in circumstances where challenge does not occur until several weeks after transfer of the X-DT cells. Mice challenged with DT cells in week 13 did not begin to express detectable diabetes until week 16, reflecting the same 3 week disease-free period observed in respect of the mice challenged in week 6. Moreover, although the period necessary for initial onset in some mice was similar for the mice challenged in week 6 and week 13, the initial rate at which the population as a whole succumbed to detectable diabetes was lower in the mice challenged in week 13 than in those challenged in week 6. In particular, 7 weeks after DT challenge, approximately 70 % of the mice challenged in week 6 suffered from detectable diabetes. In contrast, 7 weeks after DT challenge, fewer than 50 % of the mice challenged in week 13 suffered from detectable diabetes.

Thus, not only are transferred X-DT cells capable of retaining their regulatory power after prolonged exposure to the host animal, but the regulatory effect is enhanced when a moderate prechallenge period is allowed.

EXAMPLE 15 DIABETOGENIC T CELLS CULTURED IN XLCM IN THE ABSENCE OF ACCESSORY CELLS HAVE A PREVENTATIVE EFFECT AGAINST THE TRANSFER OF IDDM IN NOD.SCID MICE

Accessory cells present antigen to T-cells and may also provide co-stimulatory signals. Accessory cells are normally considered necessary to T-cell activation. The results of Example 7, as well as the shift in cytokine profile observed in XLCM culture suggested that T-cell activation was occurring in these experiments. In light of the normal role for accessory cells in T-cell activation, the effect of the co-culture of accessory cells with NOD spleen T-cells in XLCM was investigated to determine if the presence of accessory cells enhanced the effect of XLCM culture.

A) Effect of Accessory Cells on Diabetes Protection

NOD mice suffering from active diabetes were sacrificed and splenocytes were removed. A portion of these splenocytes were purified to remove accessory cells. Both splenocyte T-cell populations with (X-DT/AC) and without (X-DT) accessory cells were cultured in HBCM-2 containing 5 % XLCM for 6 days. Diabetogenic T-cells (DT) were isolated using a T-cell enrichment column (R&D Systems) on the day of adoptive transfer. T-cells were injected intraperitoneally into female NOD.Scid mice (6-8 weeks of age) (5 000 000 cells / mouse), and the mice were monitored for the onset of diabetes. The results of this experiment are depicted in Figure 15A. NOD.Scid mice not injected with cells will eventually develop diabetes because of their genetic predisposition to this disease. Injection of DT cells accelerated the onset of diabetes in these animals, while DT cells cultured in the presence of XLCM™ for 6 days (X-DT/Ac) delayed the onset of diabetes. The removal of accessory cells prior to culture (X-DT) enhanced this protective effect.

B) Effect of Accessory Cells on Cytokine Production

Splenocytes and purified T-cells were prepared from diabetic NOD mice and cultured in the presence of absence of accessory cells as described in (A), above. The splenocytes and purified T-cells were cultured in 24-well plates (200 000 cells/well) in HBCM-2 containing 5 % XLCM for five days. The cytokine profiles of these cells were measured using flow cytometry and the results are depicted in Figure 15B. These

results indicate that co-culture with non-T-cell splenocytes reduces the production of IL-4 by T-cells. (i.e. The removal of accessory cells from splenocytes prior to culture in XLCM™ increases the proportion of IL-4 producing cells in the T-cell population obtained following culture.) Thus, Th2 cytokine producing cells are reduced in the presence of non-T splenocytes.

C) Effect of Accessory Cells on the Differentiation of Diabetogenic Splenic T-cells Cultured in XLCM™

Splenocytes and purified T-cells were prepared and cultured in the presence or absence of accessory cells as described in (B), above. The differentiation of the cultured cells was assessed using flow cytometry. The results of this experiment are depicted in Figure 15 C. These results indicate that the removal of accessory cells from splenocytes prior to culture in XLCM™ results in decreased proportions of $CD4^+$ $CD8^+$ DP cells and $CD4^+$ $CD8^-$ SP cells, and an increased proportion of $CD4^-$ $CD8^+$ SP cells. The percentage of T cells recovered in the splenocyte culture is similar to that in the purified T cell culture.

EXAMPLE 16 REPOPULATION AND PROLIFERATION OF X-DT CELLS IN NOD.SCID MICE

In light of the results of Example 15, it was desirable to determine the types of T-cells which proliferated following adoptive transfer of XLCM cultured cells. Splenic T-cells were isolated from NOD.Scid mice with active diabetes and cultured for 5 - 7 days in HBCM-2 containing 5 % XLCM. These cells were then harvested and adoptively transferred into NOD.Scid mice. Mice were sacrificed at 7, 21, 35, 60, and 120 days after adoptive transfer and $CD3^+$, $CD4^+$, and $CD8^+$T-cells were quantified using three-colour flow cytometry. The results of this experiment are depicted in Figure 16. These results indicate that $CD4^+$ T-cells are preferentially expanded *in vivo* in the period following adoptive transfer of T-cells cultured in XLCM. The results demonstrate that X-DT cells have the ability to repopulate and proliferate in NOD.Scid

mice.

EXAMPLE 17 CYTOKINE PRODUCTION BY SPLEEN T CELLS OF NOD.SCID MICE ADOPTIVELY TRANSFERRED 60 DAYS PREVIOUSLY WITH X-DT CELLS

In light of the results of Examples 12, 13, 14 and 16, it was desirable to determine the cytokine profile of T-cells in mice which had been adoptively transferred with XLCM cultured T-cells, and to relate this to the development of diabetes following challenge with diabetogenic T-cells.

T-cells from NOD mice expressing active diabetes were cultured in HBCM-2 containing 5 % XLCM for a period of 5 - 6 days. These cells (X-DT) cells were then injected into NOD.Scid mice (5 000 000 cells / mouse) either alone or in conjunction with uncultured T-cells from NOD mice suffering from active diabetes (DT cells). Sixty days later, the mice were sacrificed, and splenic T-cells were removed for cytokine production analysis by flow cytometry. The results of this experiment are depicted in Figure 17. Overall cytokine production was lower among T-cells obtained from mice that received DT cells only compared to mice that received both DT and X-DT cells. The results show that IL-4 producing cells are predominant in the spleens of NOD.Scid mice adoptively transferred with X-DT cells. A higher percentage of cytokine-producing cells was found in the large T-cell compartment. The NOD.Scid mice that were co-transferred with X-DT and DT cells and developed IDDM at day 30 to 35 had a lower percentage of cytokine-producing cells.

EXAMPLE 18 CYTOKINE PRODUCTION BY MESENTERIC LYMPH NODE T-CELLS OF IDDM-FREE NOD.SCID MICE ADOPTIVELY TRANSFERRED 60 DAYS PREVIOUSLY WITH X-DT CELLS

T-cells from NOD mice with active diabetes were cultured in HBCM-2

medium containing 5 % XLCM for 5-6 days. These (X-DT) cells were harvested and introduced into NOD.Scid mice by adoptive transfer (5 000 000 cells / mouse). Sixty days later these animals were sacrificed and mesenteric lymph node cells were removed for cytokine production analysis. The results of this experiment are depicted in Figure 18. The results indicated a significant level of IL-4 producing T-cells. In particular, the results show that IL-4 producing T-cells were predominant in the lymph nodes of IDDM free NOD.Scid mice adoptively transferred with X-DT cells, and $CD8^+$ IFN-γ-producing cells were undetectable.

EXAMPLE 19 SURVIVAL AND PHENOTYPE OF ADOPTIVELY TRANSFERRED CELLS

To investigate the relationship between the survival of transferred cells and the development of diabetes, cell populations differing slightly in the Thy 1 receptor (thereby providing a marker to distinguish the cell types), but having comparable ability to reconstitute the immune system of a Scid mouse were employed.

Two populations of T-cells from NOD mice with active diabetes were prepared. The first population (Thy 1.2) was derived from NOD mice expressing the Thy1.2 membrane marker, and these cells were cultured in HBCM-2 containing 5 % XLCM for 5-6 days. The second population (Thy 1.1) was derived from NOD mice expressing the Thy 1.1 membrane protein and was not cultured in the presence of XLCM prior to transfer.

Adoptive Transfer of Thy 1.1 and Thy 1.2 Cells

NOD Scid mice were adoptively transferred with Thy 1.2 (X-DT) T-cells (5 000 000 cells / mouse). Eight weeks later they were challenged with Thy 1.1 (DT) T-cells. The percent of mice free from detectable diabetes over the observation period is depicted in Figure 19 A. Twenty weeks after the initial Thy 1.2 transfer, the mice were sacrificed and the number of T-cells of each type from various tissues was assessed.

This data is depicted in Figure 19 B, wherein PLN refers to cells obtained from the pancreatic lymph node, MLN refers to cells obtained from the mesenteric lymph node, Figure 19 B(A) depicts the total number of T-cells of both types; Figure 19 B (B) depicts the numbers of Thy1.1$^+$ T-cells, and Figure B(C) depicts the percentage of Thy1.1$^+$ cells in lymphoid tissue. The results demonstrate that the ratio of Thy1.1$^+$ (DT) cells to Thy1.1$^-$ (X-DT) cells is higher in diabetic than non-diabetic mice.

The cytokine profile of the Thy 1.1 and Thy 1.2 cell populations obtained as described above were analyzed by flow cytometry. Splenocytes were stained intracellularly with PE-conjugated anti-mouse IL-4 or anti-mouse IFN-γ in conjunction with surface staining for CD3 with FITC-conjugated anti-mouse CD3 monoclonal antibodies, and analyzed by flow cytometry. The results of this experiment are depicted in Figure 19 C. IL-4 producing cells were found to be predominant in non-diabetic NOD.Scid mice that were adoptively pre-transferred with X-DT cells and then challenged with DT cells.

Diabetes was first detected in some mice 3 weeks after challenge with Thy 1.1 DT cells. By 12 weeks after Thy 1.1 DT cell challenge, approximately half the mice had developed detectable diabetes. Diabetic mice had lower overall T-cell counts than did non-diabetic mice. The percentage of Thy 1.1 cells in relation to total T-cells was higher in diabetic mice than in non-diabetic mice, although non-diabetic mice had greater total numbers of Thy 1.1 cells than did diabetic mice. Thus, the absolute numbers of diabetogenic T-cells do not appear to be determinative of disease development. Instead, the relative levels of protective and diabetogenic T-cells appears to be important. T-cells from non-diabetic mice expressed higher levels of IL-4 than did T-cells from diabetic mice.

EXAMPLE 20 T-CELLS ISOLATED FROM NON-DIABETOGENIC NOD.SCID MICE ADOPTIVELY TRANSFERRED WITH X-DT CELLS ARE NOT PATHOGENIC

The ability of XLCM cultured cells to regulate the development of diabetes upon transfer of a mixed cell population to a second mouse was examined.

A first population of T-cells was isolated from NOD mice suffering from active diabetes. These cells were cultured in XLCM for 6 days (generating X-DT cells) and then adoptively transferred into NOD.Scid mice. Sixty days later these NOD.Scid mice were challenged by the transfer of uncultured T-cells from diabetic NOD mice (DT cells). Some recipient mice developed diabetes while others were protected. Sixty days after the DT cell challenge, T-cells were isolated from the spleens of the diabetic and non-diabetic mice which had received the first cell population and the challenge. These cells were transferred directly to NOD.Scid mice and the incidence of diabetes in the second set of recipients was monitored. The procedure employed is illustrated in Figure 20 A. The results of this experiment are depicted in Figure 20 B.

T-cells obtained from non-diabetic mice did not cause detectable diabetes in the recipient mice during the 15 week observation period. T-cells from diabetic mice caused diabetes in all recipient mice within 7 weeks after transfer. Thus, XLCM culture is capable of rendering diabetogenic T-cells non-diabetogenic and this effect persists through transplantation into a second animal. Further, XLCM culture is capable of inducing the cultured T-cells to exert a protective effect rendering injected DT cells non-diabetogenic, suppressing diabetes, and this effect persists through transplantation into a second animal.

EXAMPLE 21 THE IMPACT OF CULTURE IN XLCM ON THE DIABETOGENIC AND IDDM PREVENTATIVE TENDENCIES OF ISOLATED CD4 AND CD8 CELLS

Spleens were removed from diabetic NOD mice, and $CD4^+$ cells were isolated according to standard procedures. The isolated $CD4^+$ cells were cultured in HBCM-2 medium containing 5 % XLCM™ in the absence or presence of IL-4 (5 ng/ml) for 4 days. The resultant cell population was adoptively transferred (5 000 000 cells per

mouse) into NOD.Scid mice. A portion of the mice were challenged by the transfer of a population of uncultured diabetogenic cells (DT cells) at 0 or 7 days after the initial transfer of X-DT cells. The mice were observed for 18 weeks and the onset of diabetes was noted. The results of this experiment are depicted in Figure 21 A. The same basic procedure was repeated using isolated $CD8^+$ cells with the effect of DT cell challenge at day 0, week 1, and week 8 examined. The results of this experiment are depicted in Figure 21 B.

The transfer of $CD4^+$ X-DT cells alone lead to the onset of diabetes in approximately 70 % of the mice within 5 weeks of transfer and no further incidence was noted in the 18 week observation period. Where $CD4^+$X-DT cells were cultured in the presence of exogenous IL-4, the rate of diabetes after 5 weeks was approximately 60 %, with no increase during the observation period.

The transfer of $CD8^+$ X-DT cells alone did not lead to the onset of diabetes within the 18 week observation period. Moreover, $CD8^+$ X-DT cells had a protective effect against diabetogenesis by DT "challenge" cells that increased with the time intervals between injection of $CD8^+$ X-DT cells and DT challenge. These results are depicted in Figure 21 B.

While it is not intended that the scope of the invention should be interpreted or limited by any particular theory or postulation of its mode of action, the following discussion is offered for a more complete understanding of the invention as a whole.

It is believed that the maturation from DP to SP thymocytes is initiated by interaction of the TcR on DP thymocytes with peptide bound to MHC molecules expressed on thymic stromal cells. However, since DP thymocytes may differentiate *in vitro* into either $CD4^+CD8^-$ or $CD4^-CD8^+$ SP thymocytes in the absence of thymic stromal cells, the differentiation of DP to SP thymocytes may not strictly require an interaction between TcR/co-receptor and peptide-MHC complexes. In agreement with

these findings, XLCM induces the proliferation and differentiation of DP into $CD4^+CD8^-$ and $CD4^-CD8^+$ SP thymocytes in the absence of thymic stromal cells. Unlike other culture systems in which thymocytes must be activated by stimulation via the TcR/CD3 complex, the differentiation of thymocytes in XLCM does not appear to require such stimulation. XLCM consists of multiple factors, e.g. various cytokines, chemokines and growth factors, which may provide the requisite signals to induce thymocyte proliferation and differentiation and obviate the need for TcR activation. Although IL-2 is present in XLCM and is required for XLCM-driven T cell proliferation, it is unlikely that IL-2 is solely responsible for the support of thymocyte proliferation and differentiation, since addition of exogenous IL-2 to various culture media does not substitute efficiently for the growth stimulatory properties of XLCM.

In the thymus, some DP thymocytes may already be precommitted to the CD4 or CD8 lineage by default, and further selection of a given lineage may be mediated by cytokines. Thus, an alternative explanation for the effect of XLCM on thymocyte maturation is that some of the cytokines present in XLCM may drive precommitted DP thymocytes to differentiate into $CD4^+CD8^-$ or $CD4^-CD8^+$ SP thymocytes by down-regulation of the CD8 or CD4 co-receptors, respectively. This possibility is supported by the observations that CP and IL-4 each alter the pattern of thymocyte differentiation, albeit in an opposite fashion, when added to XLCM. CP stimulates a marked increase in the growth of $CD4^-CD8^+$ SP thymocytes and decrease in the growth of $CD4^+CD8^-$ SP thymocytes, whereas IL-4 enhances the growth of $CD4^+CD8^-$ SP and $CD4^-CD8^-$ DN thymocytes but reduces the growth of $CD4^+CD8^+$ DP and $CD4^-CD8^+$ SP thymocytes. These altered patterns of differentiation were observed for thymocytes from three different mouse strains (C57BL/6, NOD and NOR), and appear to be IL-4-specific as the IL-4 triggered thymocyte differentiation was completely abrogated in the presence of an anti-IL-4 mAb. The finding is consistent with that observed in a 3 day fetal thymus organ culture system, in which IL-4 treatment increases the growth of $CD4^+CD8^-$ SP thymocytes and decreases the growth $CD4^+CD8^+$ DP thymocytes. An important difference between the XLCM and 3 day thymus organ culture systems is that, in XLCM, IL-4 directly acts on DP thymocytes in the absence of

stromal cells to deliver signals for $CD4^+$ SP thymocyte differentiation. Thus, in XLCM, CP and IL-4 can provide signals that either up- or down-regulate the expression of CD8 and CD4, respectively.

Although IL-4 stimulates the growth of $CD4^+CD8^-$ SP thymocytes, the mechanism by which IL-4 regulates the positive selection of these SP thymocytes remains unclear. In accordance with the correlation between increased surface expression of CD69 on thymocytes and positive thymic selection, we found that IL-4 significantly enhances CD69 expression on C57BL/6, NOD and NOR thymocytes cultured in XLCM, and on $CD4^+CD8^+$ DP thymocytes in particular. This result, together with the finding that anti-IL-4 mAb blocks the IL-4 induced elevation of CD69 expression on DP thymocytes, suggests that DP thymocytes are the major cell subpopulation in the thymus that is activated by IL-4.

Several strain-related differences were detected in the proliferation and differentiation of NOD and C57BL/6 thymocytes cultured in XLCM. First, while C57BL/6 $CD4^-CD8^+$ SP thymocytes were predominant in the initial 4 day cultures, NOD $CD4^+CD8^-$ SP thymocytes were more abundant in these cultures. Second, a higher percentage of $CD69^+$ cells was obtained after a 4-6 day culture of NOD than C57BL/6 thymocytes. Third, addition of IL-4 to XLCM increased the frequency of IL-4- and IL-10-producing C57BL/6 but not NOD thymocytes. Interestingly, thymocytes from NOR mice, which are much more genetically similar to NOD than C57BL/6 mice, displayed a pattern of differentiation akin to that of NOD thymocytes. These results suggest that both genetic background and environmental factors in the thymus determine thymocyte differentiation.

Thymocytes differentiate in XLCM to produce IL-2, IL-4, IL-10 and IFN-γ, and the percentages of IL-4 and IL-10 producing thymocytes were highest in these cultures. Since XLCM contains little IL-4, other factor(s) present in XLCM likely promote IL-4 secretion. Exogenous IL-4 stimulates the synthesis and secretion of IL-4 and IL-10 by thymocytes cultured in XLCM. In contrast, virtually complete inhibition

of IL-4 and IL-10 production is observed upon addition of CP, even though CP simultaneously promotes thymocyte proliferation. These results suggest that the ability of thymocytes to produce cytokines may be determined by environmental factors in the thymus, which in turn may influence thymocyte differentiation, as suggested above.

In conclusion, XLCM is a unique conditioned medium capable of inducing the proliferation and differentiation of specific T-cell subtypes *in vitro* in the absence of thymic stromal cells, and therefore represents an excellent growth medium for exploring factors that have an important impact on thymocyte differentiation. By using this process, we have obtained additional supportive evidence that IL-4 plays an important role in thymocyte differentiation, which is characterized by the differentiation of $CD4^+CD8^-$ SP thymocytes and an increase in surface CD69 expression on DP thymocytes. XLCM may be used to generate regulatory T cells, which may have important implications for cell therapy of several immunological disorders, including HIV-1 infection, cancer and autoimmune diseases including IDDM.

CLAIMS:

1. A process for *ex vivo* proliferation of T-cells and differentiation thereof to produce cell populations enriched in pre-selected sub-populations of T-lymphocytes, which comprises *ex vivo* culturing T-cells in a culture medium containing a cell suspension conditioned culture medium CM, and under appropriate cell proliferation conditions, and obtaining in the culture medium a cell population enriched in the preselected sub-population.

2. The process of claim 1 wherein the culture medium is an aqueous culture medium containing a minor proportion by volume of said conditioned culture medium CM.

3. The process of claim 2 wherein the conditioned culture medium CM is the aqueous liquid product resulting from the culturing therein of a cell population comprising peripheral blood cells, umbilical cord blood cells, bone marrow cells, mixtures of two or more types of such cells, or fractions or mixed fractions of such types of cells, in the presence of an inducing agent which includes at least one plant mitogen.

4. The process of claim 3 wherein the medium CM derives from culturing said cells in the presence of at least one plant mitogen selected from concanavalin A, phytohemagglutinin, mezerein and tetradecanoyl phorbol acetate.

5. The process of claim 4 wherein the plant mitogens used in preparation of the medium CM are concanavalin A and mezerein.

6. The process of claim 5 wherein a starting population of DP thymoyctes CD4+ CD8+ T-cells is cultured in the presence of conditioned medium CM to produce cell populations enriched in predetermined single positive CD4+ T-cells, CD8+ T-cells or sub-populations of such cells.

7. The process of claim 6 wherein DP thymocytes are cultured in the presence of

conditioned medium CM and in the presence of added exogenous IL-4 to promote differentiation thereof to SP thymocytes.

8. The process of claim 7 wherein the culturing is conducted in the substantial absence of thymic stromal cells.

9. The process of claim 5 wherein T-cells are cultured in a culture medium containing conditioned medium CM to produce a cell population enriched in IL-4 producing cells.

10. The process of claim 5 wherein self-reactive T-cells are cultured in a culture medium containing conditioned medium CM to produce a cell population with a reduced level of self-reactivity.

11. The process of claim 5 wherein T-cells are cultured in a culture medium containing conditioned medium CM to produce a cell population capable of delaying the onset of autoimmune diabetes when introduced into an MHC compatible patient.

12. The process of claim 9 wherein the starting T-cells are immature T-cells.

13. The process of claim 9 wherein the starting T-cells are mature T-cells.

14. The process of claim 9 wherein the culture medium also contains exogenous IL-4.

15. An IL-4 secreting population of mammalian T-cells containing a substantial proportion of a sub-population of Th2-like cells exhibiting a Th2-like cell cytokine production profile, said IL-4 secreting T-cell population having been prepared by cell culturing of a starting cell population containing T-cells, said starting population having a lower Th2-like cell cytokine production profile said than said IL-4 secreting T-cell population.

16. An IL-4 secreting cell population as defined in claim15, having a higher Th2-like cell cytokine production profile than its precursor cell population before culturing thereof in conditioned medium CM described herein.

17. An IL-4 secreting cell population as defined in claim 16, having a higher Th2-like cell cytokine production profile than its precursor cell population before culturing in the presence of XLCM.

18. A process of enhancing the protection of a mammal against the onset and/or development, or alleviating the symptoms, of a Th1/Th2 related cytokine disorder, which comprises administering to the mammal a population of MHC compatible T-cells.

19. The process of claim 18 wherein the cell population is obtained by the process of claim 10 or claim 11

20. The process of claim 18 wherein the cell population is as claimed in 14, claim 15 or claim 16.

21. The process of claim 18 wherein the enriched cell population is obtained by a process as claimed in claim 9.

22. The process of claim18, claim 19, claim 20 or claim 21, wherein the process is a T-cell mediated autoimmune disease.

23. The process of claim 21 wherein the autoimmune disease is IDDM.

24. The use of a population of cells produced by the process of claim 9, claim 10 or claim 11 in the preparation of a medicament for treating an MHC compatible subject against the onset and/or development of an autoimmune disease.

25. Use of a population of a self reactivity suppressing mammalian T-cells , in treating

an MHC compatible mammal for protection against the onset and/or development of, or alleviation of the symptoms of, a T-cell mediated autoimmune disorder.

26. Use of a population of mammalian T-cells enriched in IL-4 producing cell sub-population, in treating an MHC compatible mammal for protection against the onset and/or development of, or alleviation of the symptoms of, a Th1/Th2 related cytokine response disorder.

27. Use according to claim 26 wherein the disorder is an autoimmune disease.

28. Use according to claim 26 wherein the disorder is IDDM.

29. Use according to claim 26, claim 27 or claim 28 wherein the cell population is as defined in claim 15, claim 16 or claim 17.

30. Use according to claim 26 wherein the IL-4 producing enriched cell sub-population has been obtained by a process as claimed in claim 9.

Figure 1. XLCM™ Supports Thymocyte Proliferation

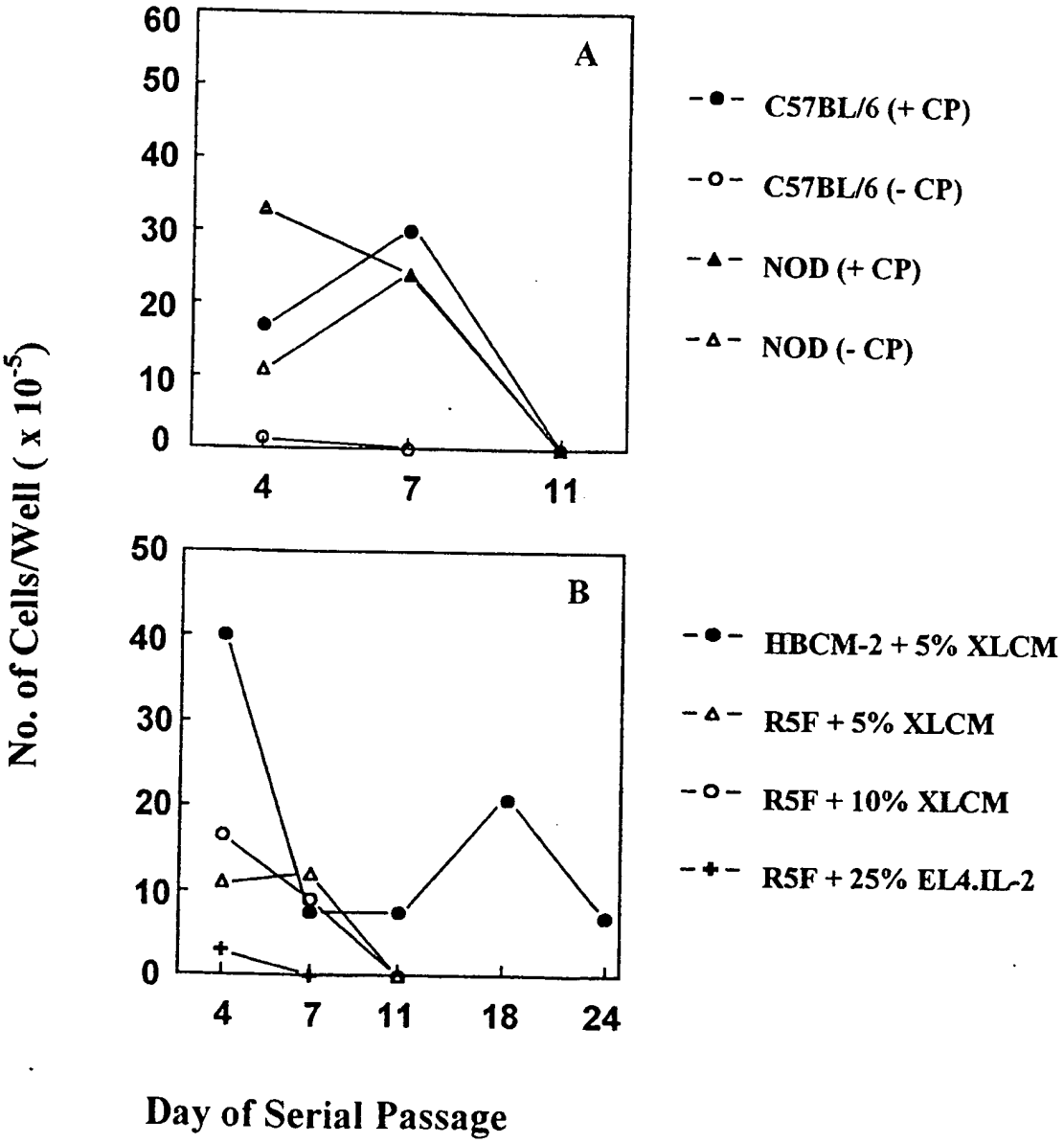
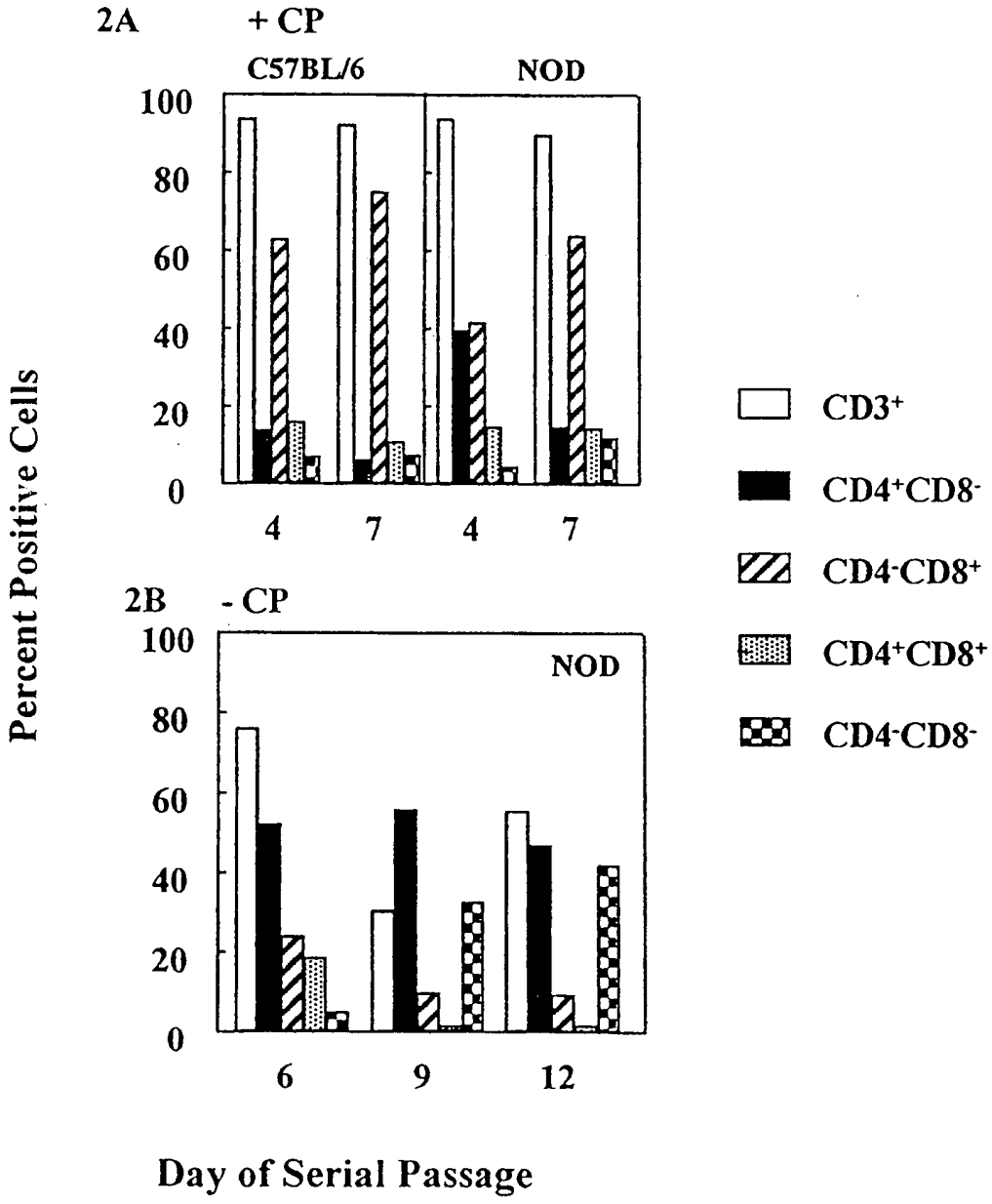


Figure 2 XLCM™ Promotes Thymocyte Differentiation



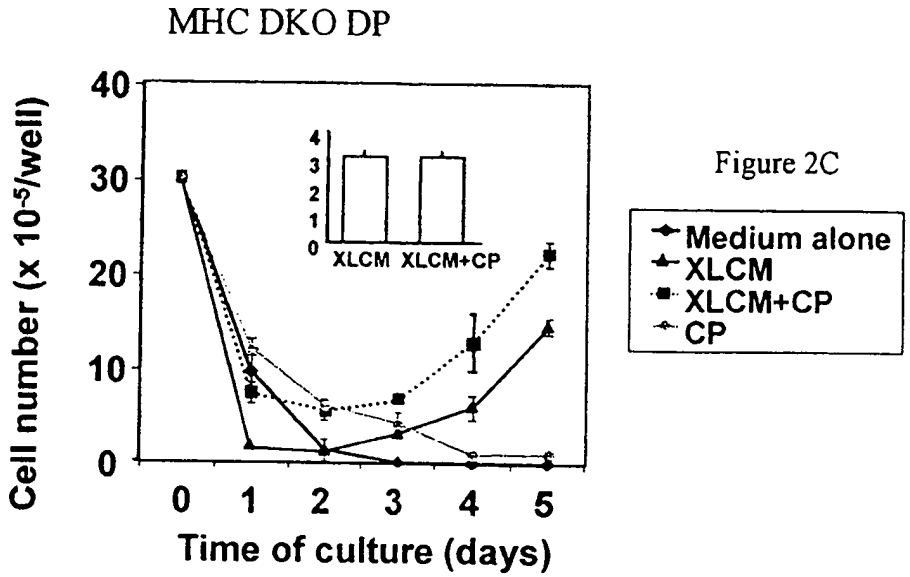


Figure 2C

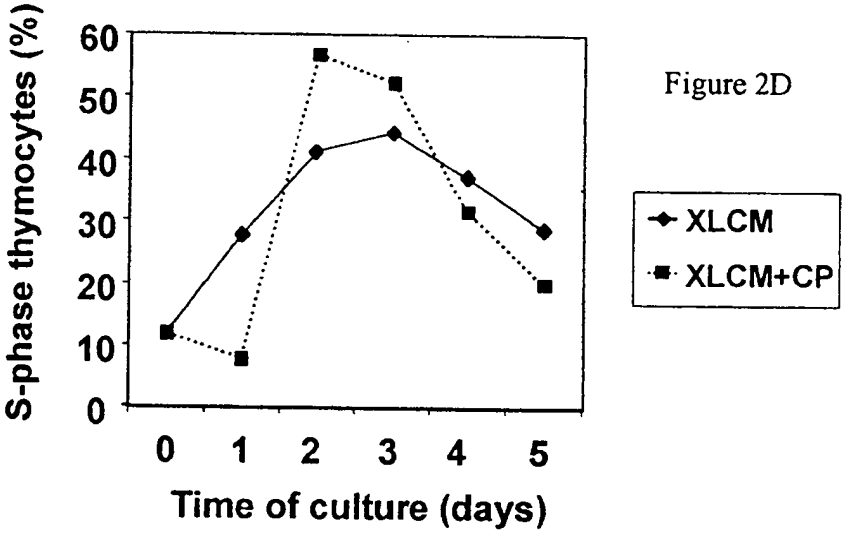


Figure 2D

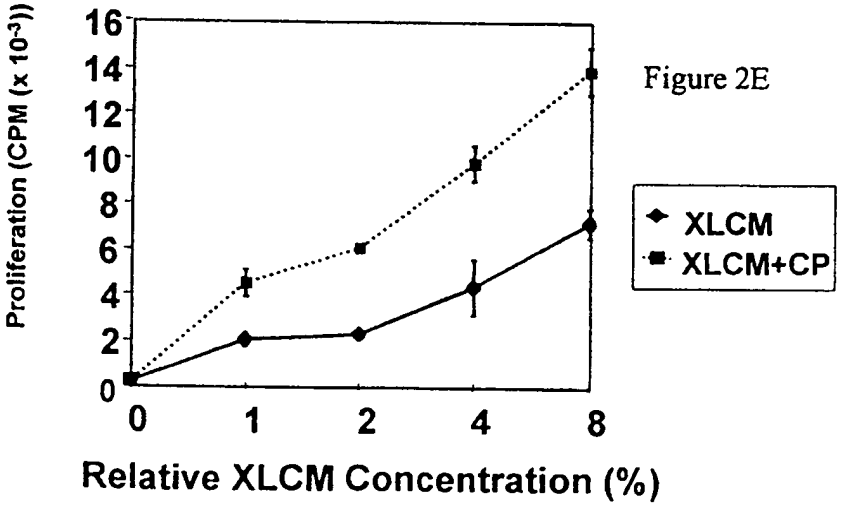


Figure 2E

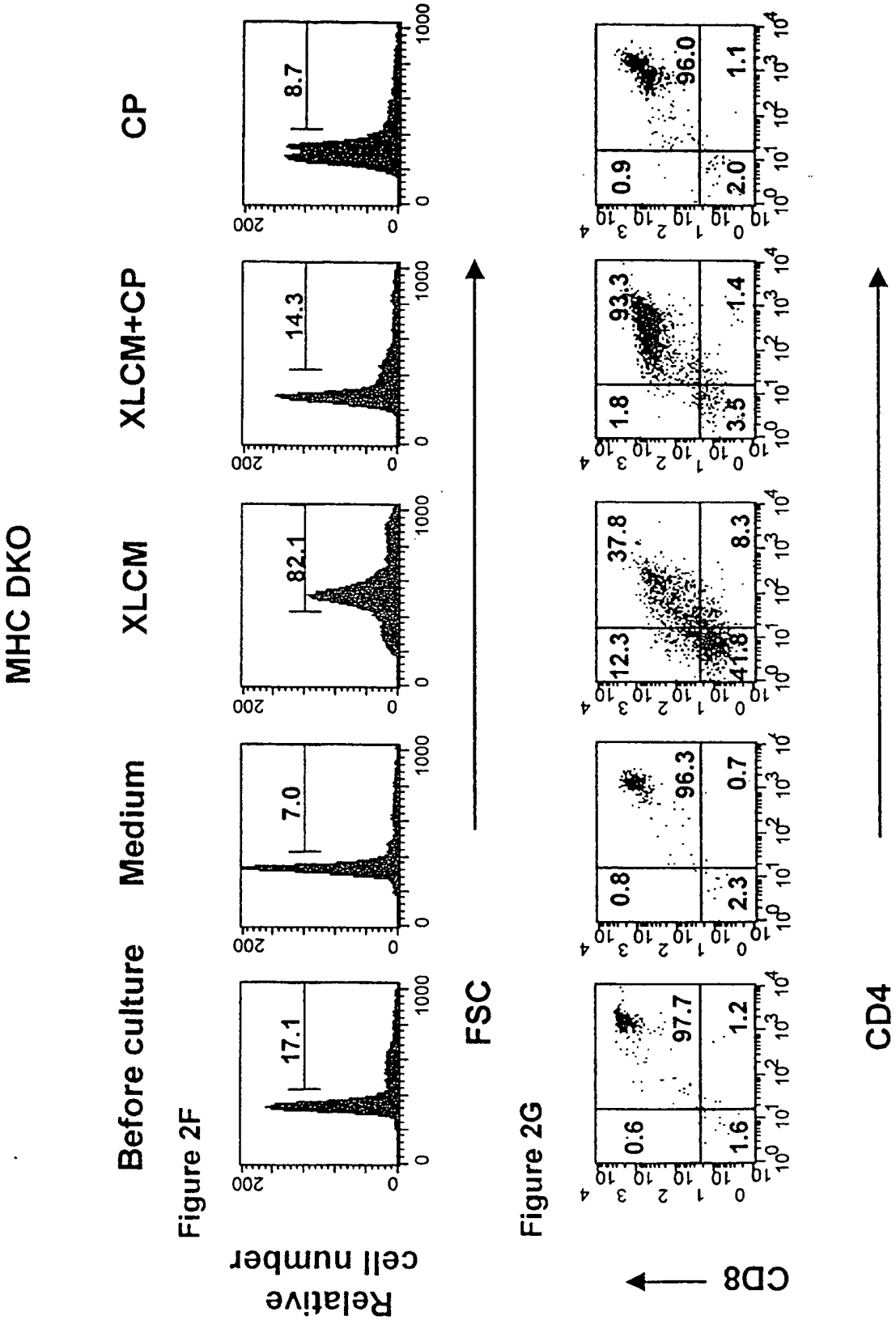


Figure 2F

Figure 2G

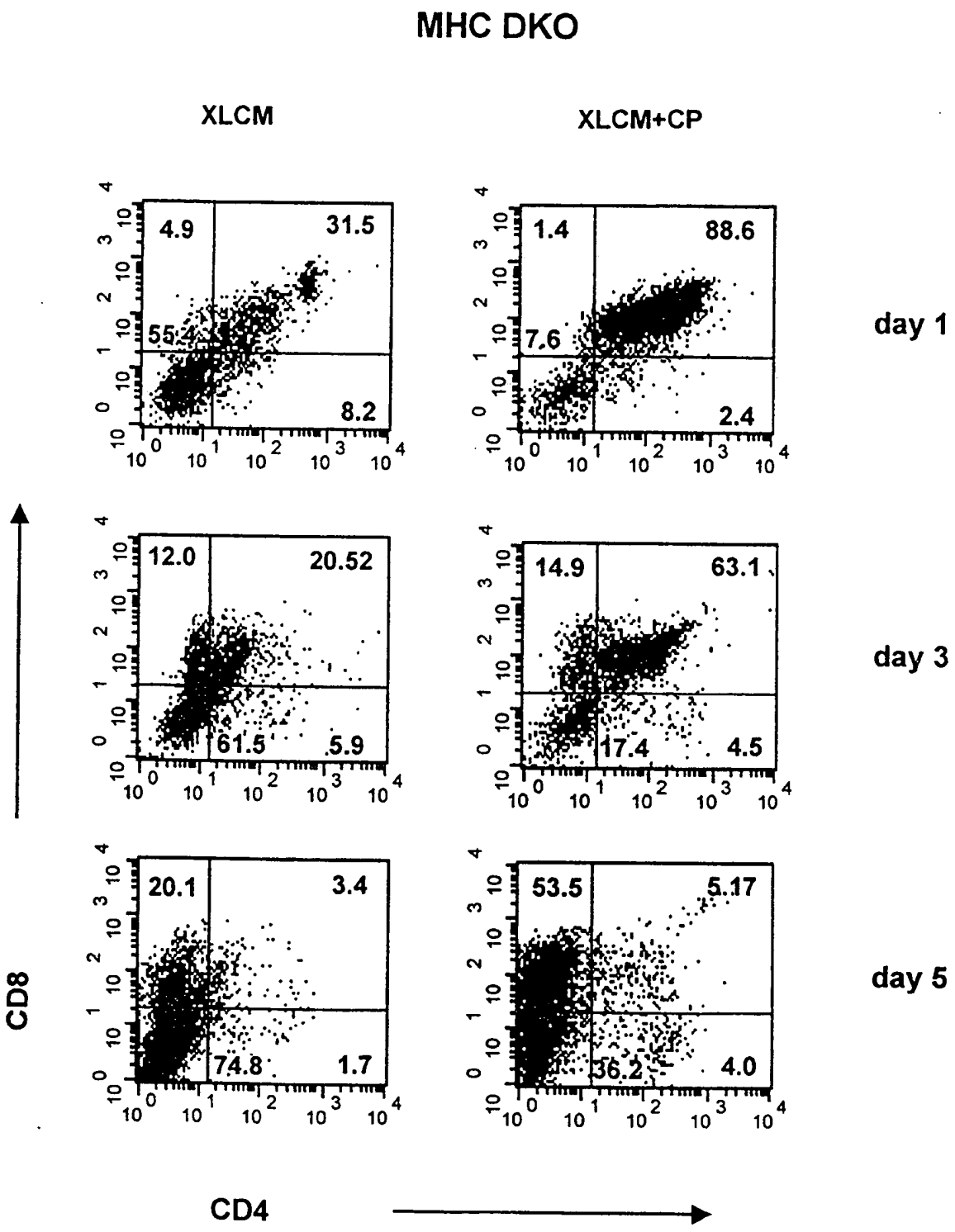

MHC DKO
XLCM
XLCM+CP
4.9
31.5
55.4
8.2
1.4
88.6
7.6
2.4
day 1
12.0
20.52
61.5
5.9
14.9
63.1
17.4
4.5
day 3
20.1
3.4
74.8
1.7
53.5
5.17
36.2
4.0
day 5
CD8
CD4

Figure 2H

Figure 3A

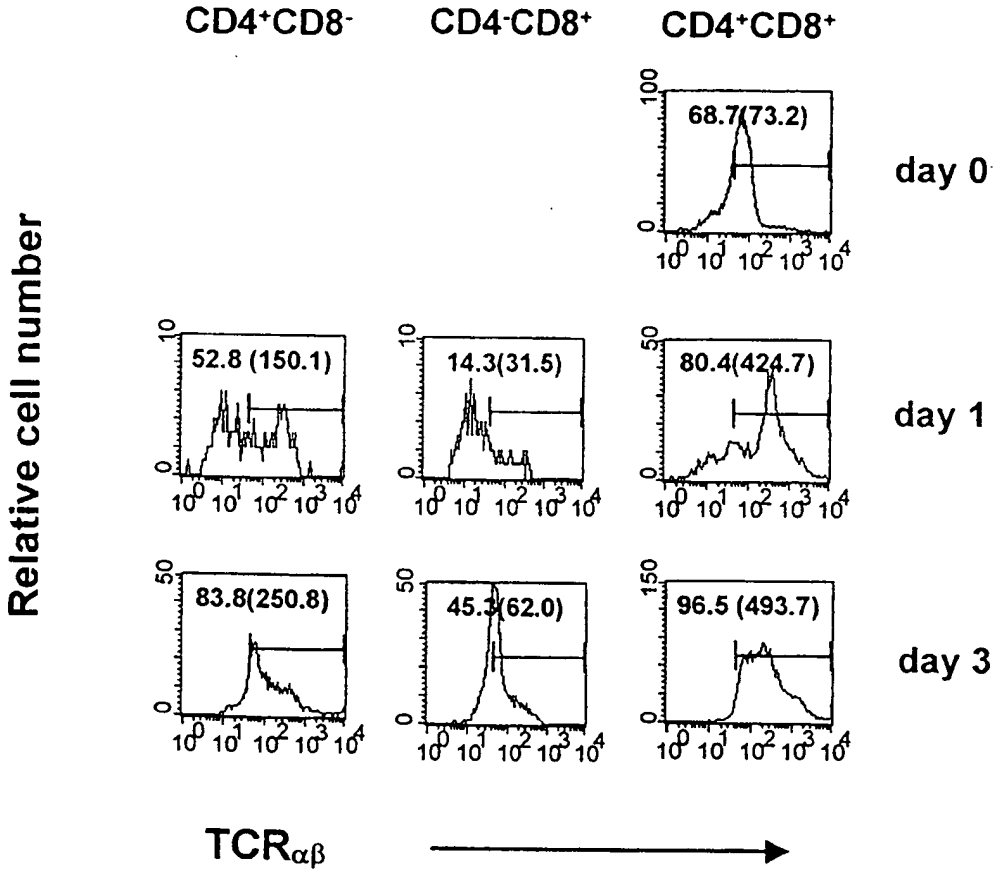

CD4+CD8-
CD4-CD8+
CD4+CD8+
Relative cell number
68.7(73.2)
day 0
52.8 (150.1)
14.3(31.5)
80.4(424.7)
day 1
83.8(250.8)
45.3(62.0)
96.5 (493.7)
day 3
TCRαβ

Figure 3B

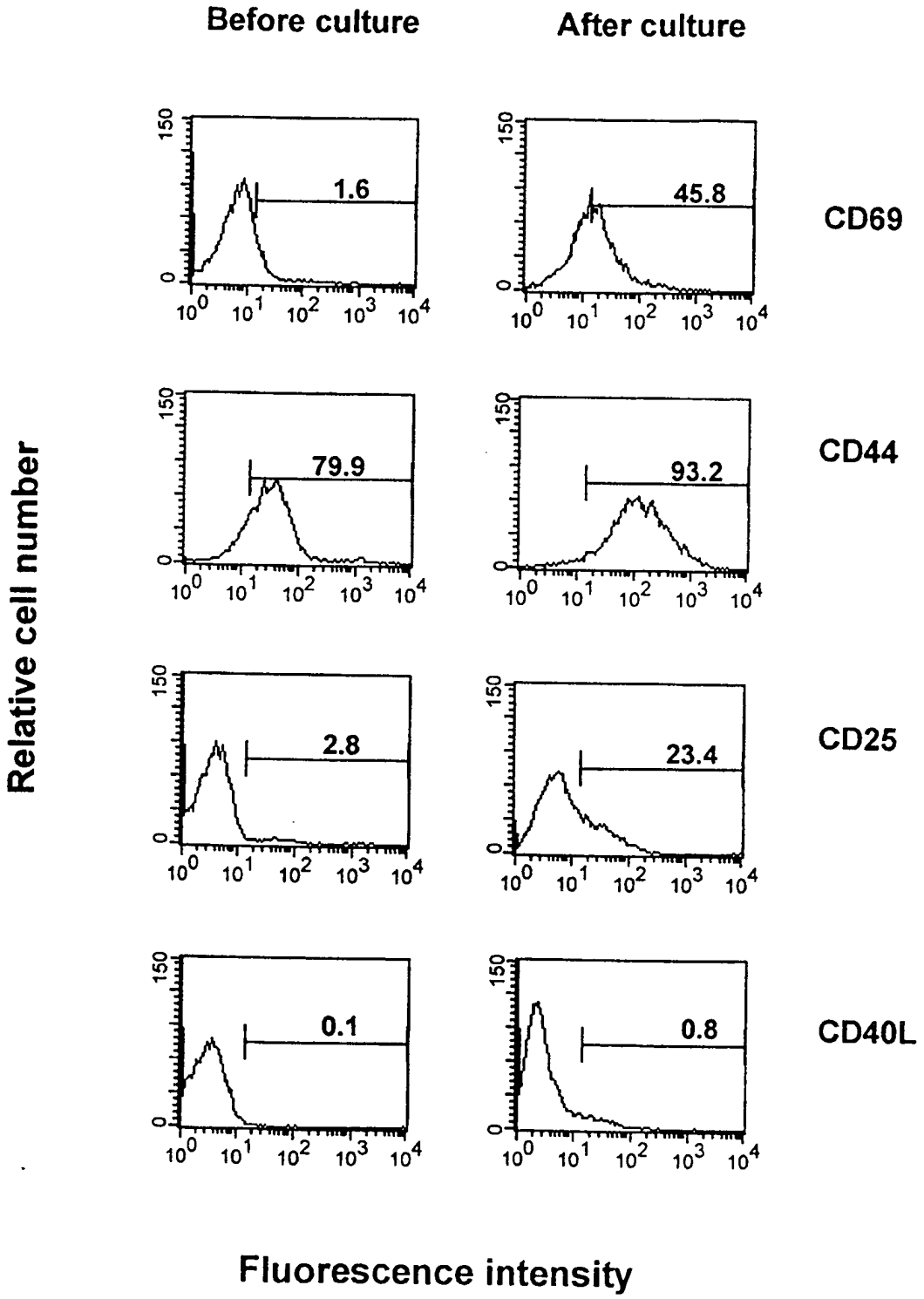

Before culture
After culture
1.6
45.8
CD69
79.9
93.2
CD44
2.8
23.4
CD25
0.1
0.8
CD40L
Relative cell number
Fluorescence intensity

Figure 4A

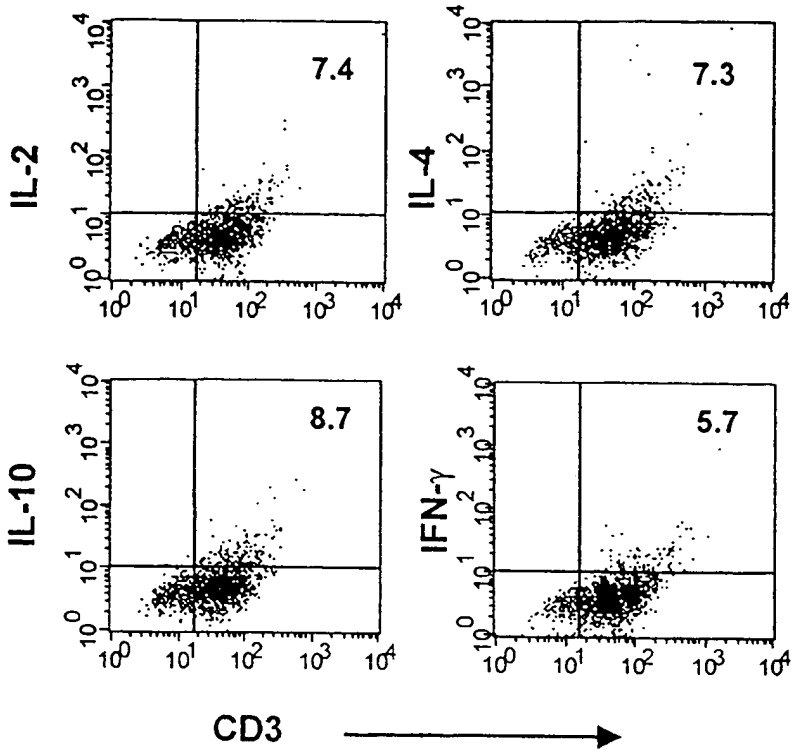


Figure 4B

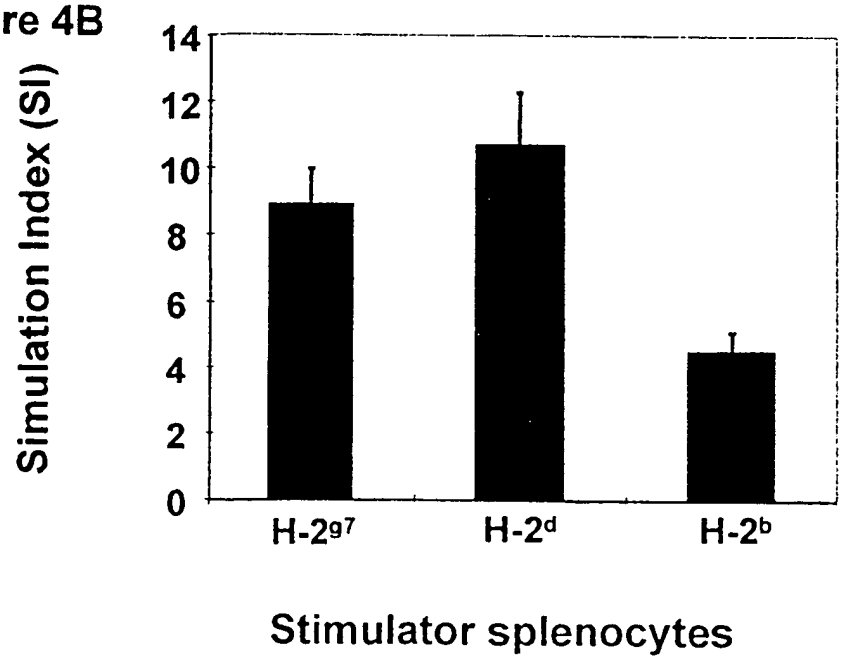
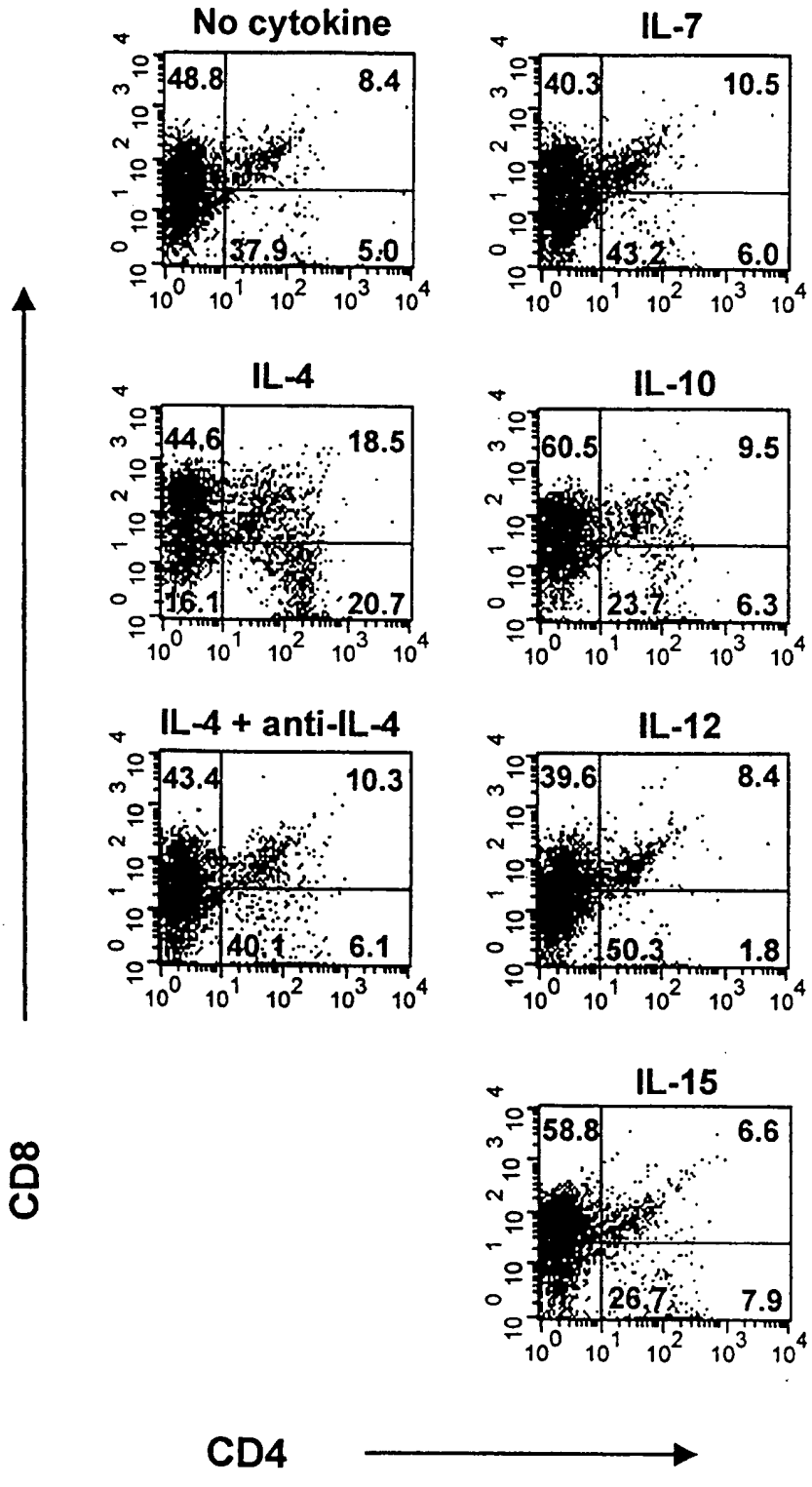


Figure 5A


No cytokine
48.8
8.4
37.9
5.0
IL-7
40.3
10.5
43.2
6.0
IL-4
44.6
18.5
16.1
20.7
IL-10
60.5
9.5
23.7
6.3
IL-4 + anti-IL-4
43.4
10.3
40.1
6.1
IL-12
39.6
8.4
50.3
1.8
IL-15
58.8
6.6
26.7
7.9
CD8
CD4

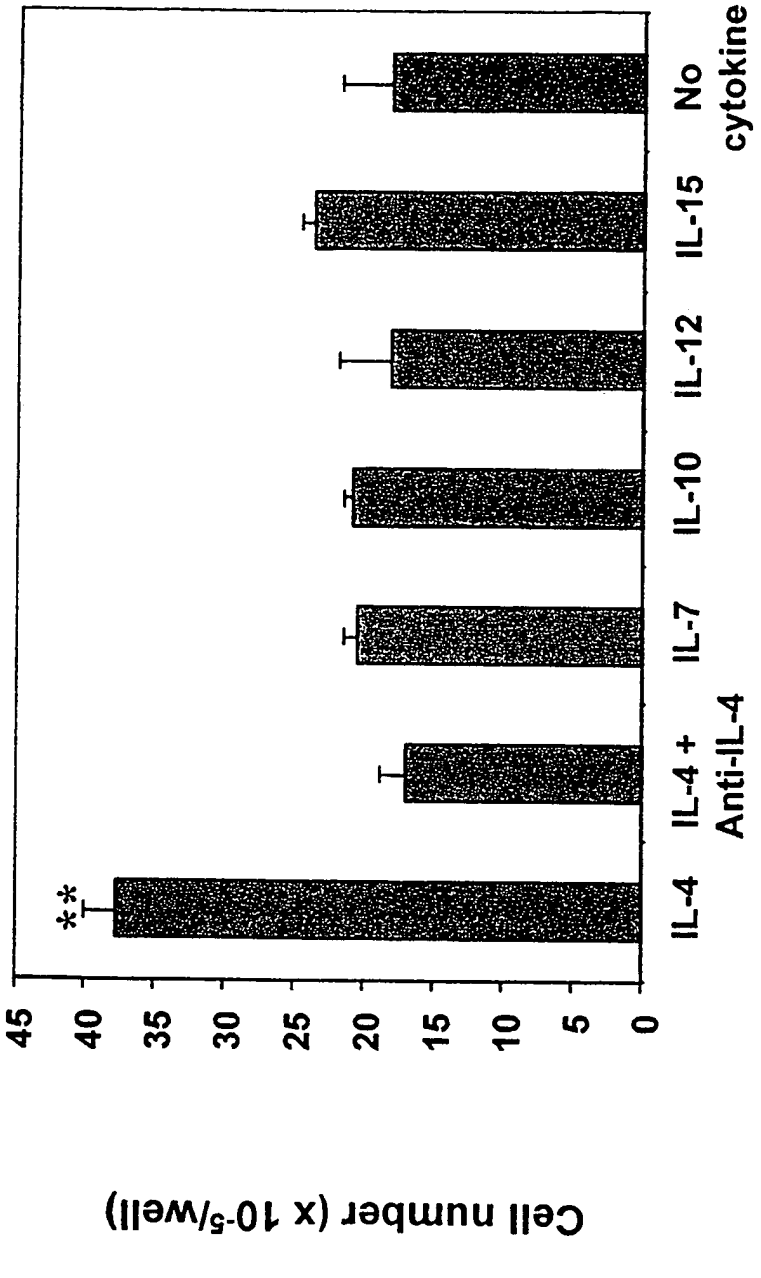

B
Cell number (x 10-5/well)
45
40
35
30
25
20
15
10
5
0
**
IL-4
IL-4 + Anti-IL-4
IL-7
IL-10
IL-12
IL-15
No cytokine

Figure 5B

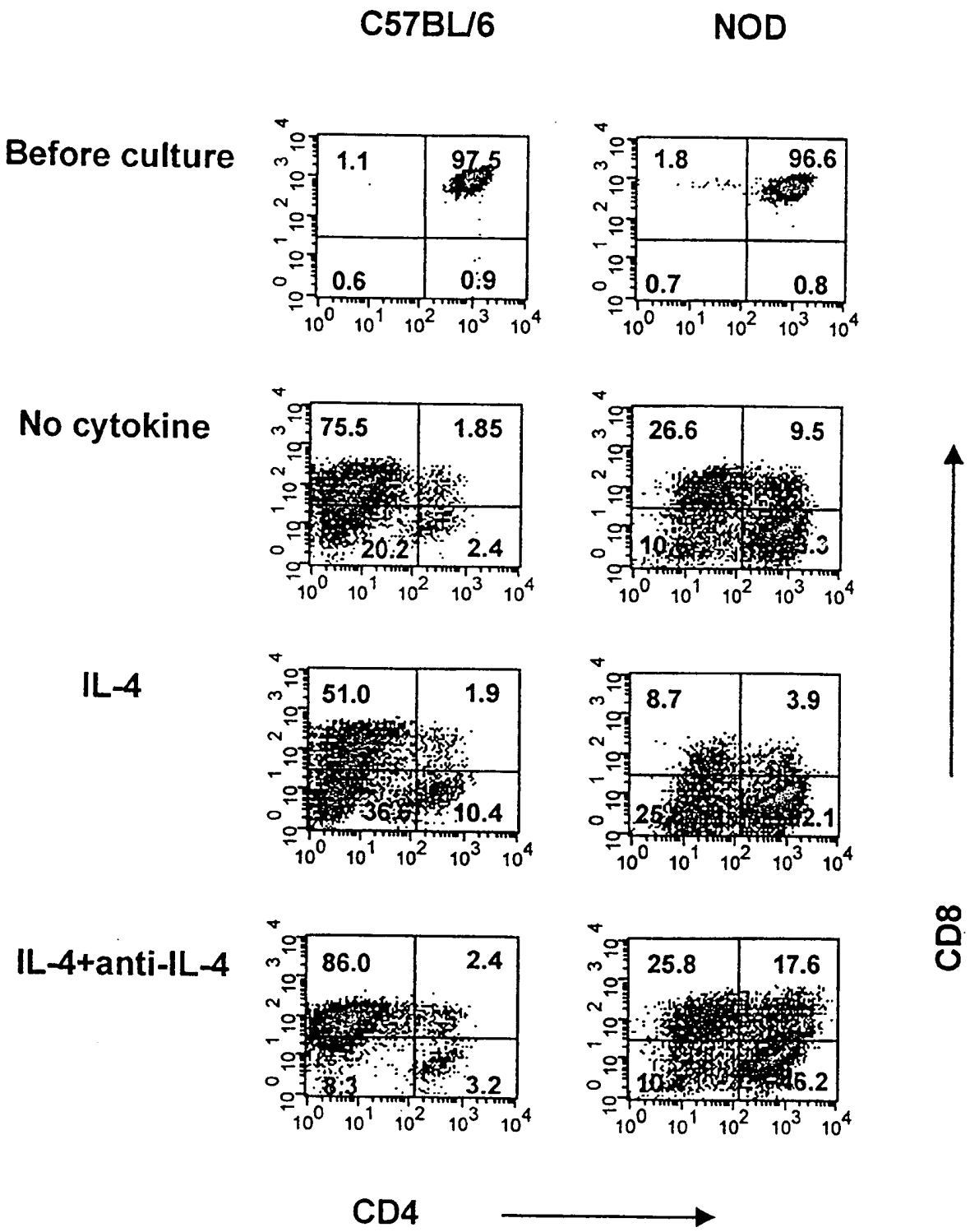

C57BL/6
NOD
Before culture
No cytokine
IL-4
IL-4+anti-IL-4
1.1
97.5
0.6
0.9
1.8
96.6
0.7
0.8
75.5
1.85
20.2
2.4
26.6
9.5
51.0
1.9
36.6
10.4
8.7
3.9
86.0
2.4
3.3
3.2
25.8
17.6
CD4
CD8

Figure 6

Figure 7A IL-4 Upregulates CD69 Expression

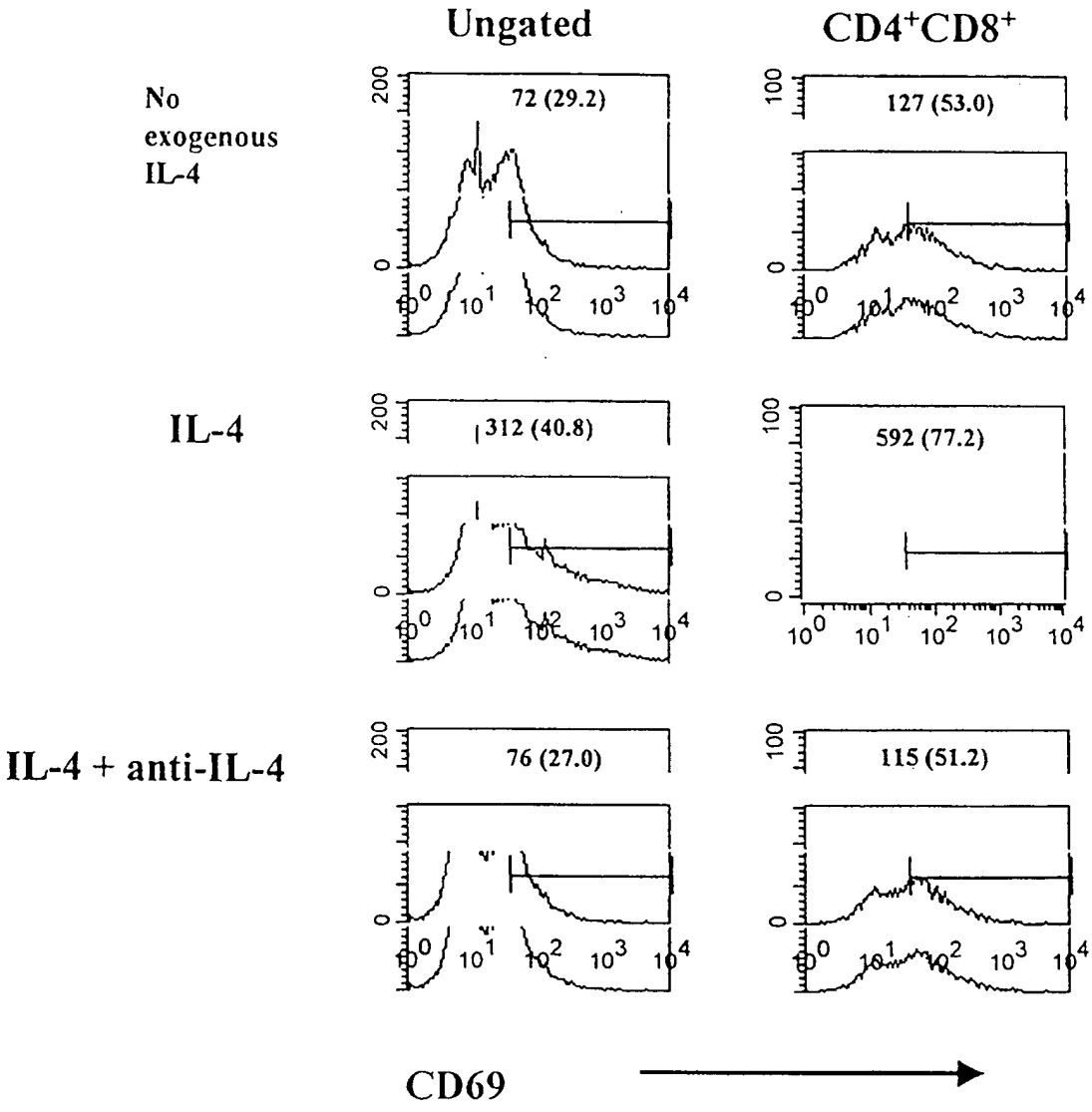


Figure 7B IL-4 Upregulates CD69 Expression

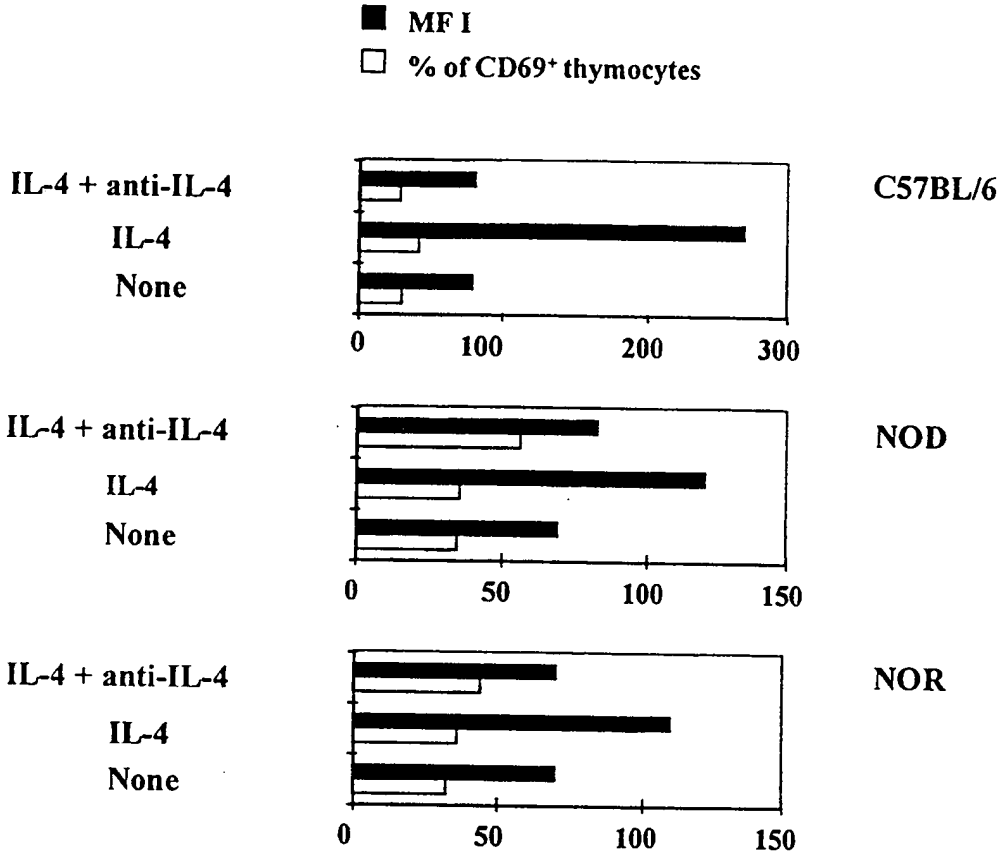


Figure 8A

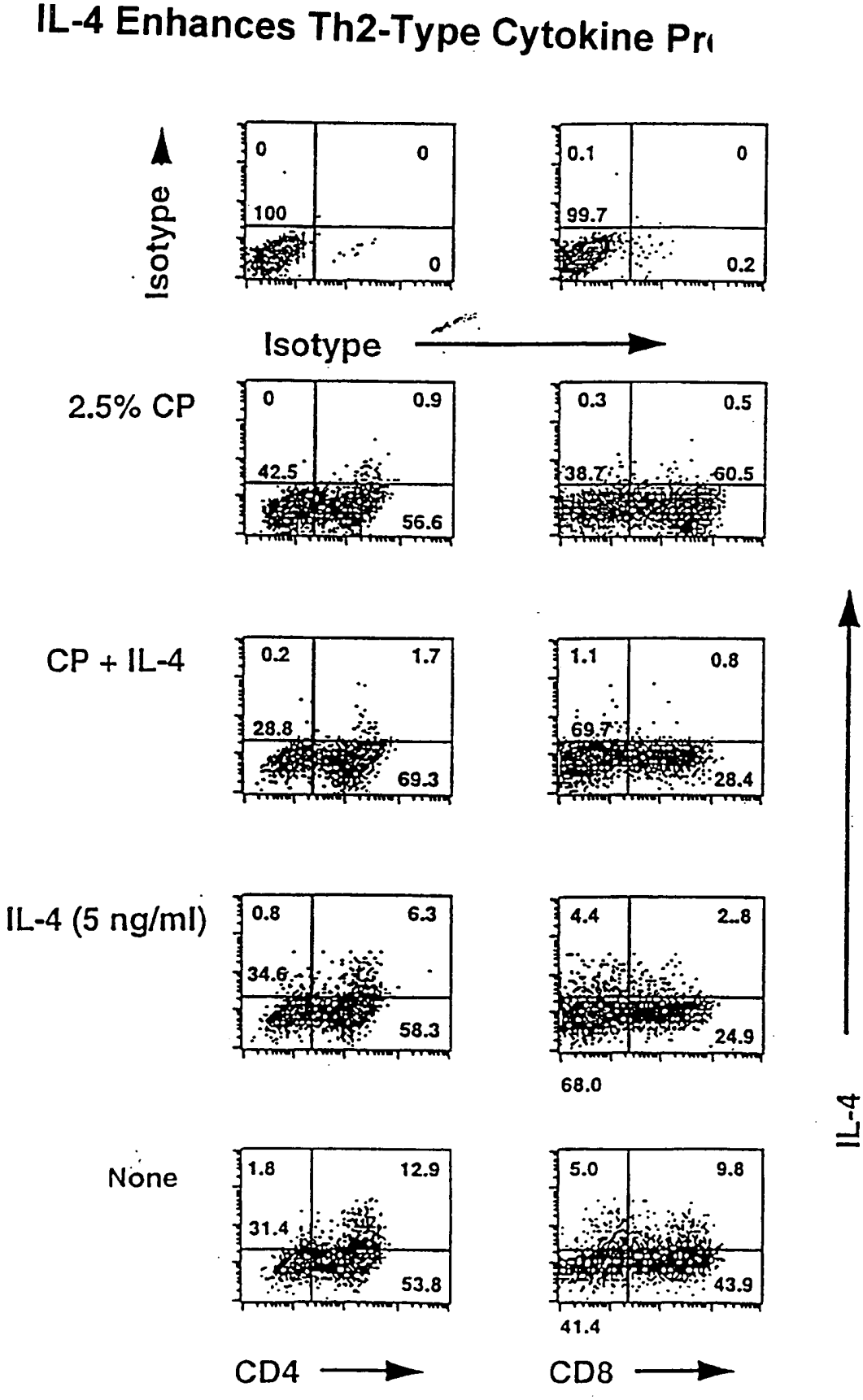

IL-4 Enhances Th2-Type Cytokine Pr
Isotype
Isotype
2.5% CP
CP + IL-4
IL-4 (5 ng/ml)
None
0
0
100
0
0.1
0
99.7
0.2
0
0.9
42.5
56.6
0.3
0.5
38.7
60.5
0.2
1.7
28.8
69.3
1.1
0.8
69.7
28.4
0.8
6.3
34.6
58.3
4.4
2..8
24.9
68.0
1.8
12.9
31.4
53.8
5.0
9.8
43.9
41.4
IL-4
CD4
CD8

Figure 8B IL-4 Enhances Th2-Type Cytokine Production

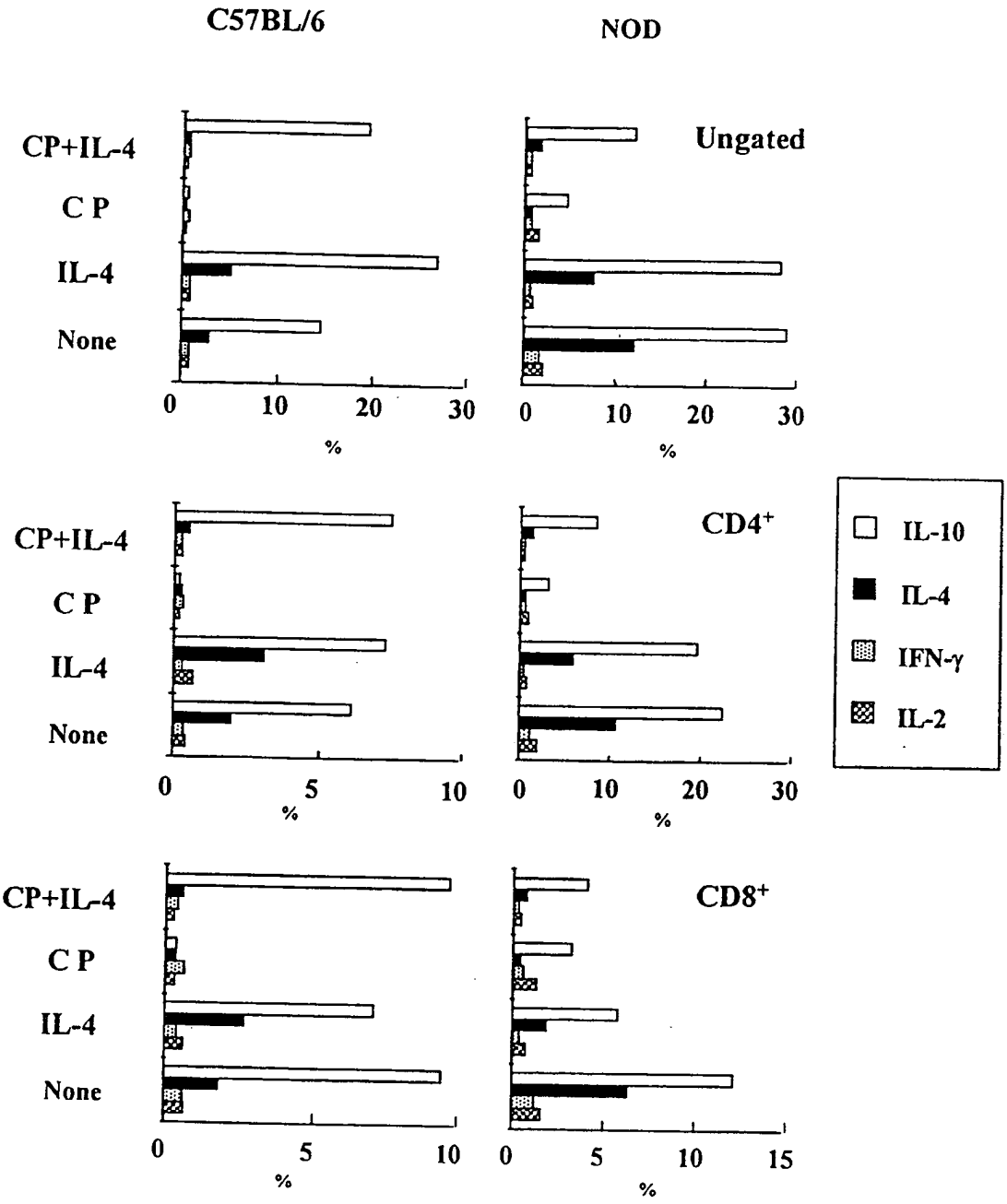


Figure 9 XLCM™ Stimulates Splenic T Cell Proliferation

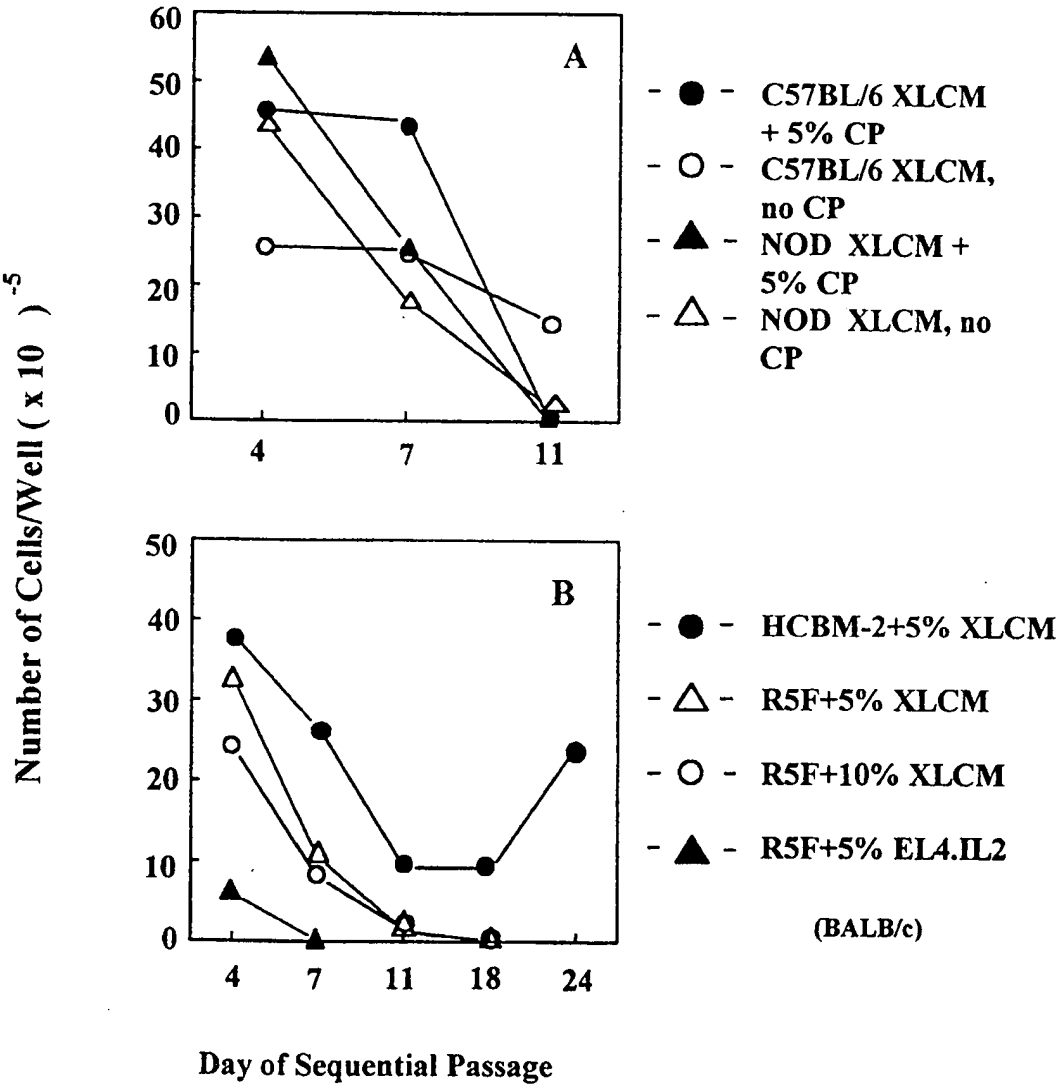


Figure 10 Phenotype of Splenocytes Expanded in XLCM™

Figure 10A

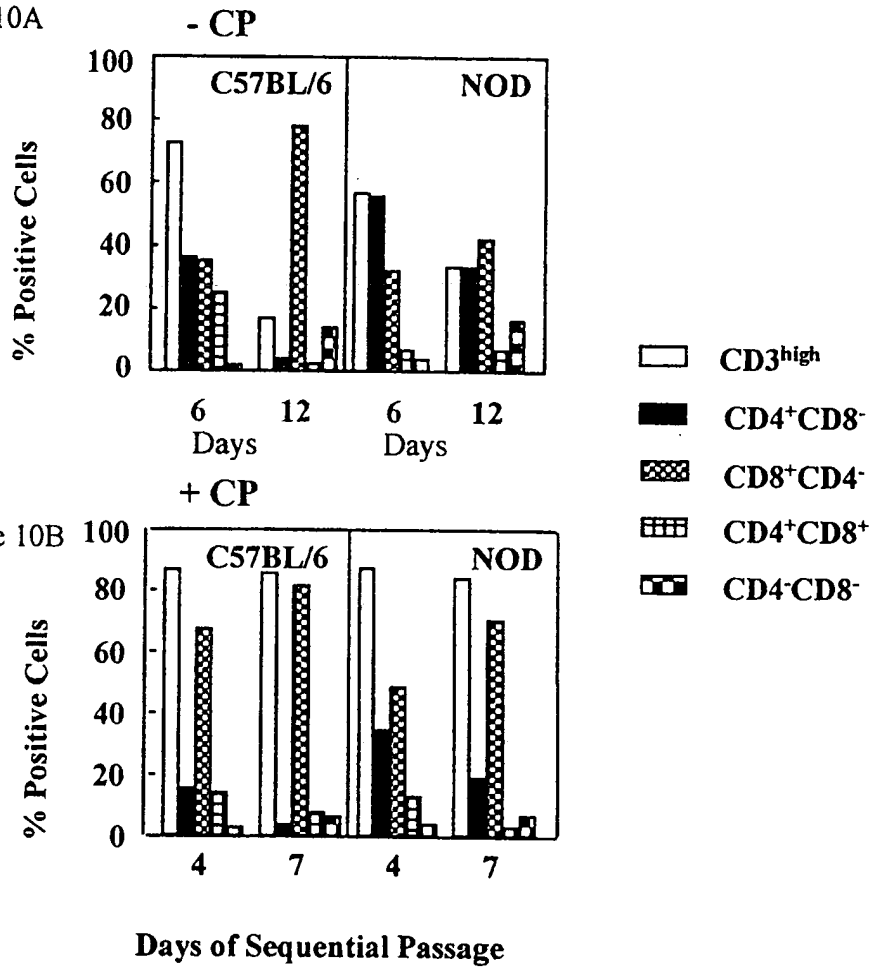


Figure 10B

Figure 11 IL-4 Enhances Th2-Like Cells

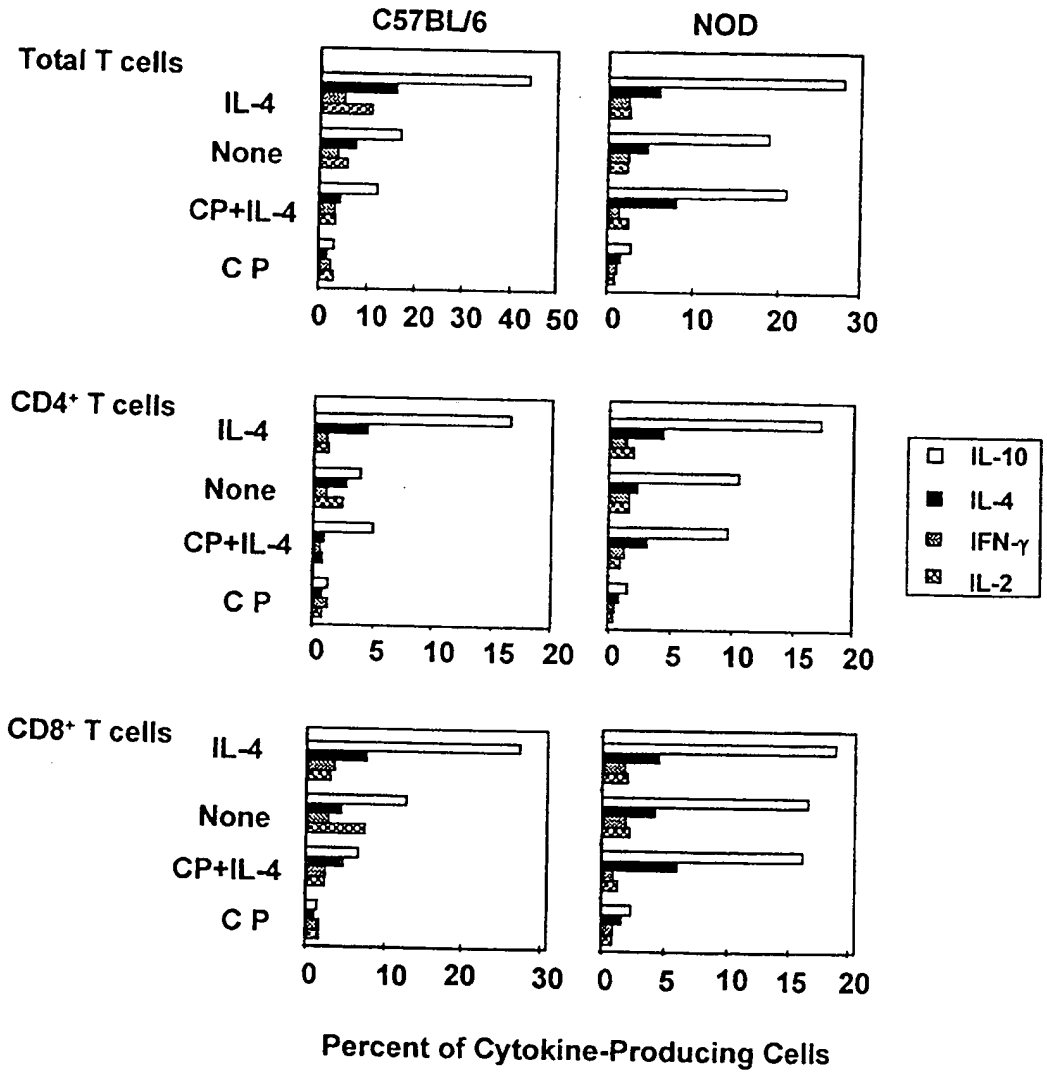


Figure 12 Th2 Cytokine Profile Promotion

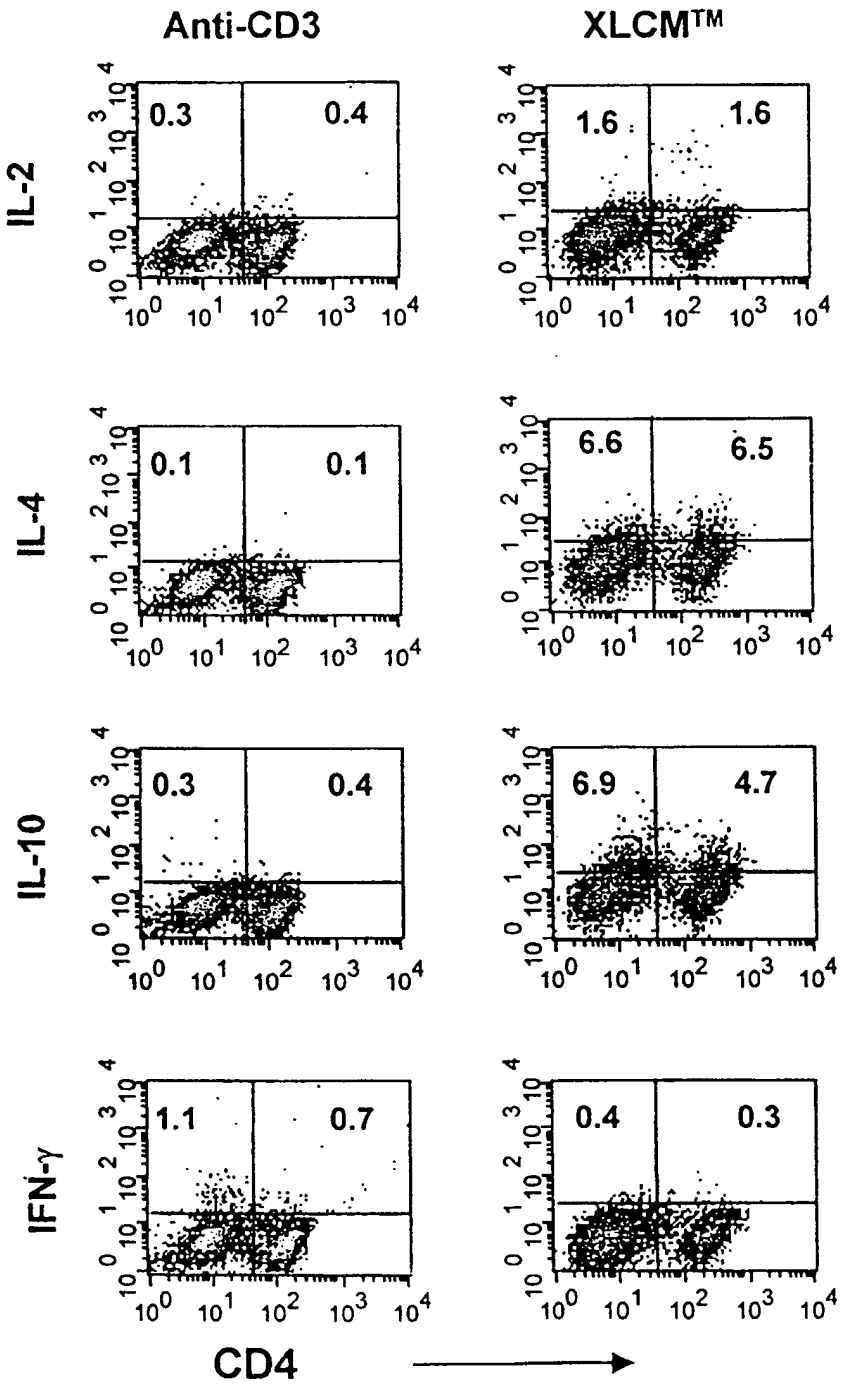


Figure 13 Reduced Diabetogenesis

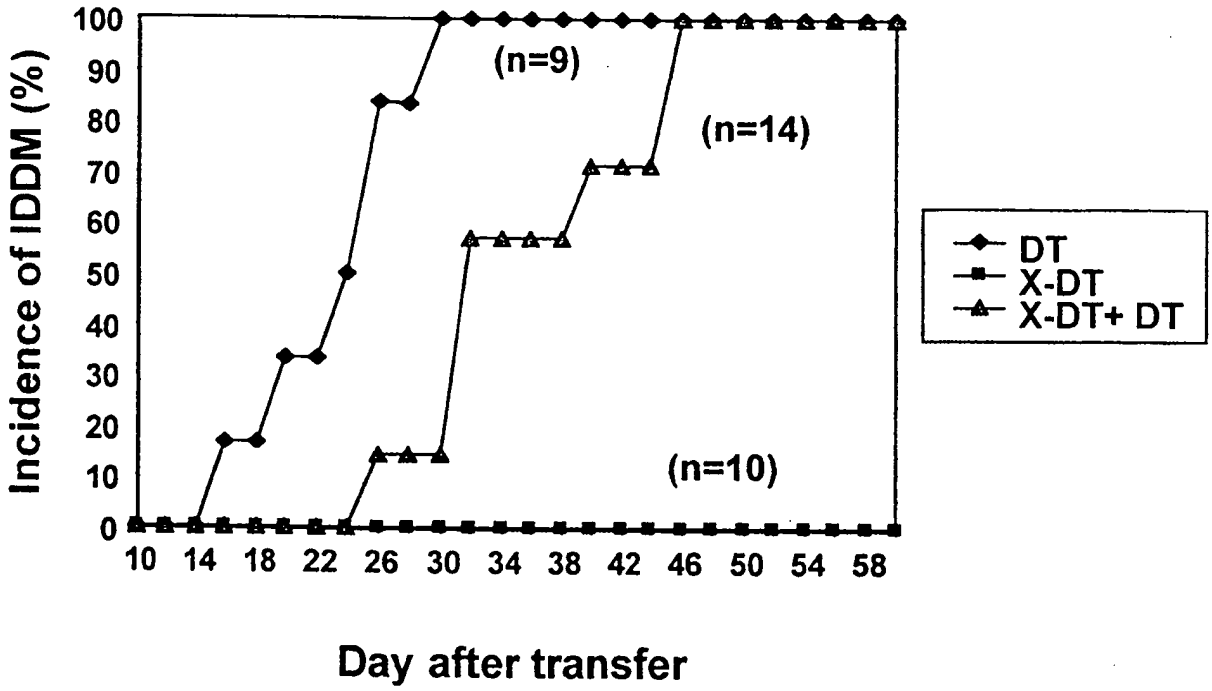


Figure 14 Kinetics of Protection

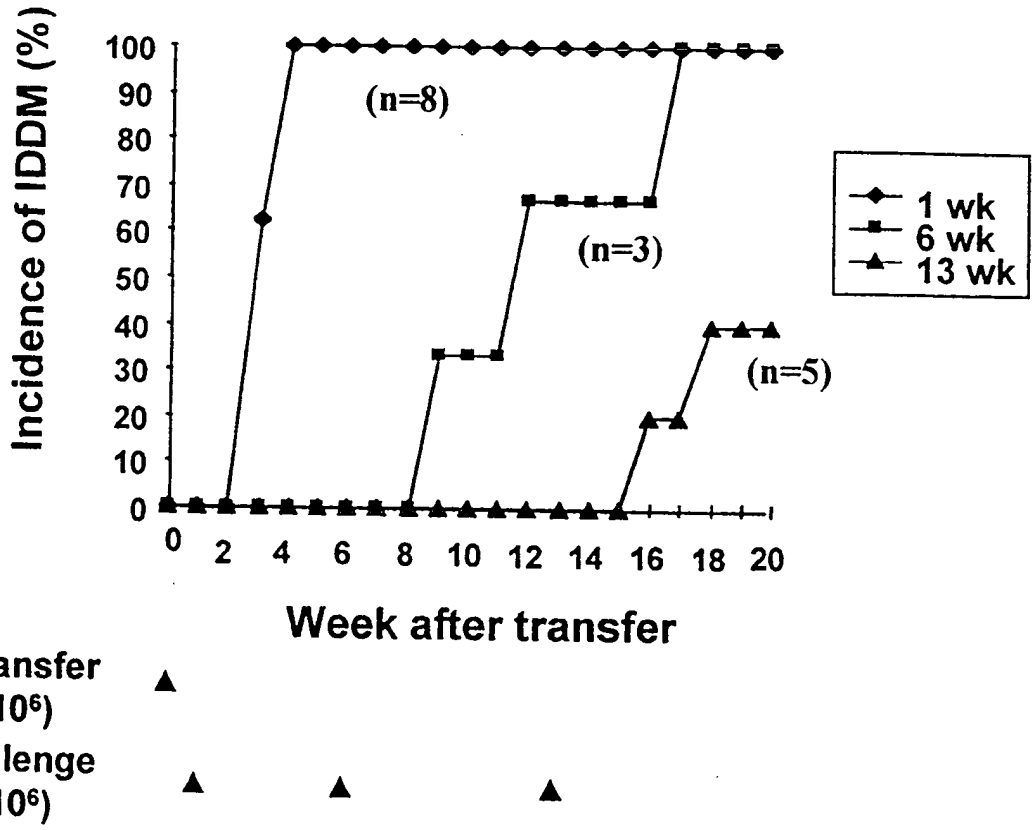


Figure 15A Accessory Cell Culture and IDDM

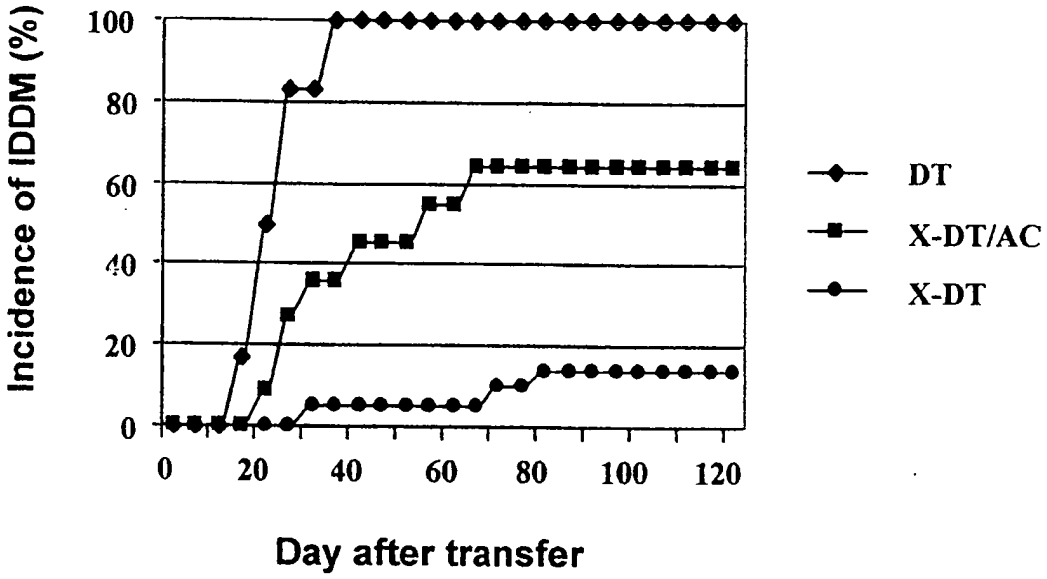


Figure 15B Accessory Cell Culture and Cytokine Production

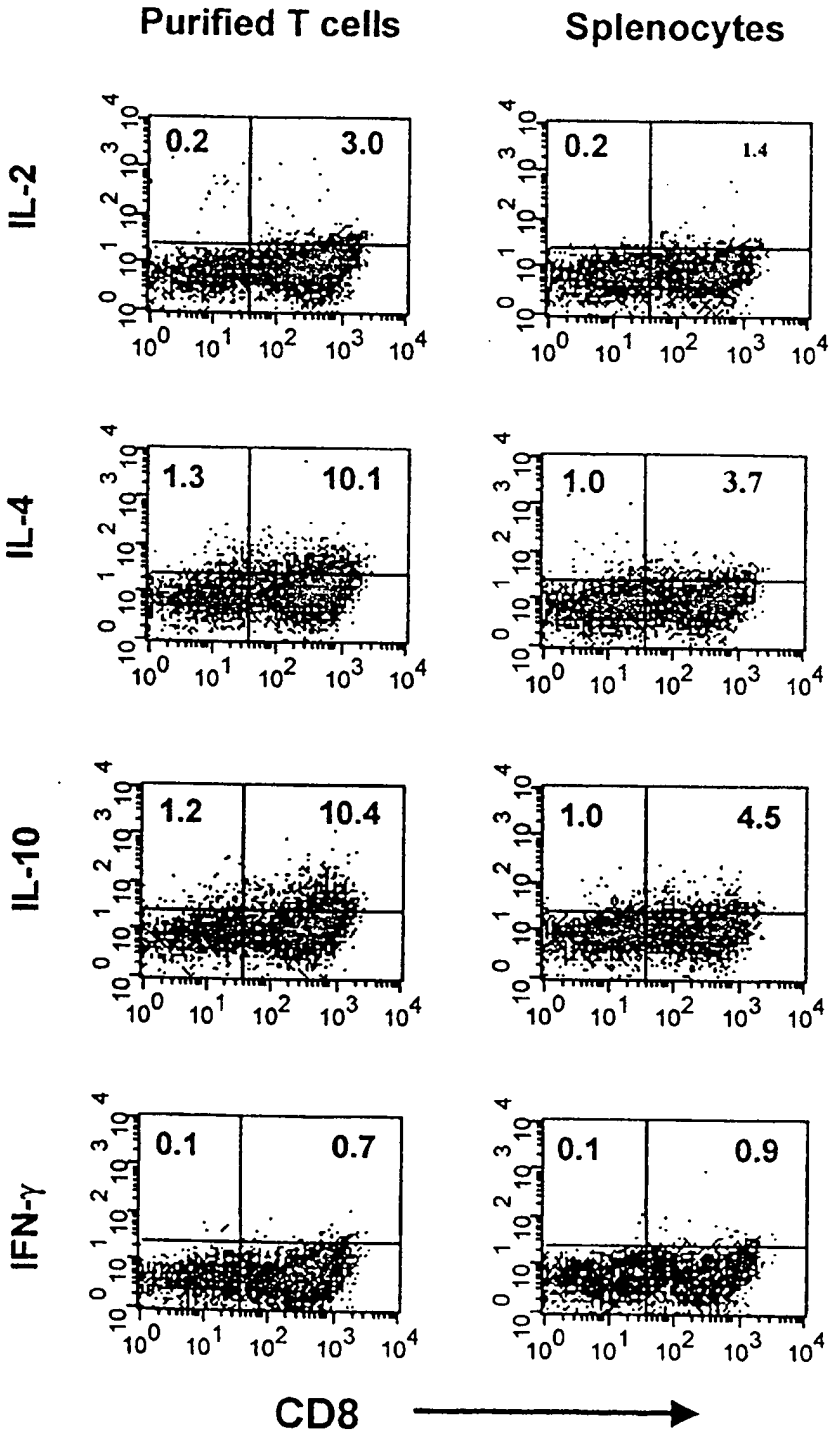


Figure 15C Accessory Cells and CD4, CD8 Expression

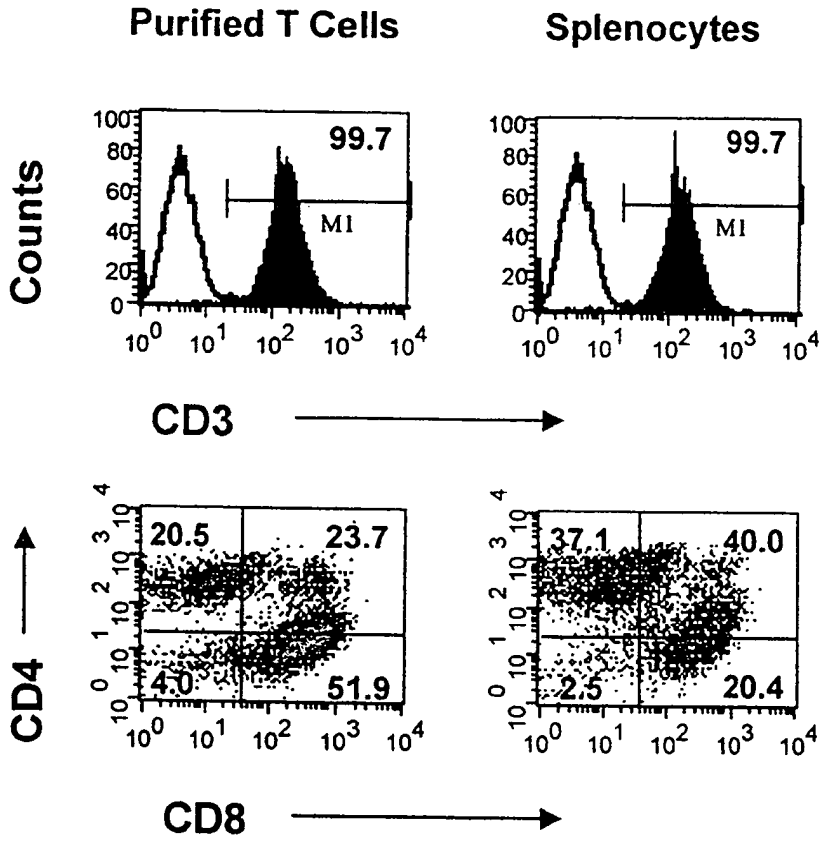


Figure 16 Repopulation and Proliferation of X-DT Cells in NOD.Scid Mice

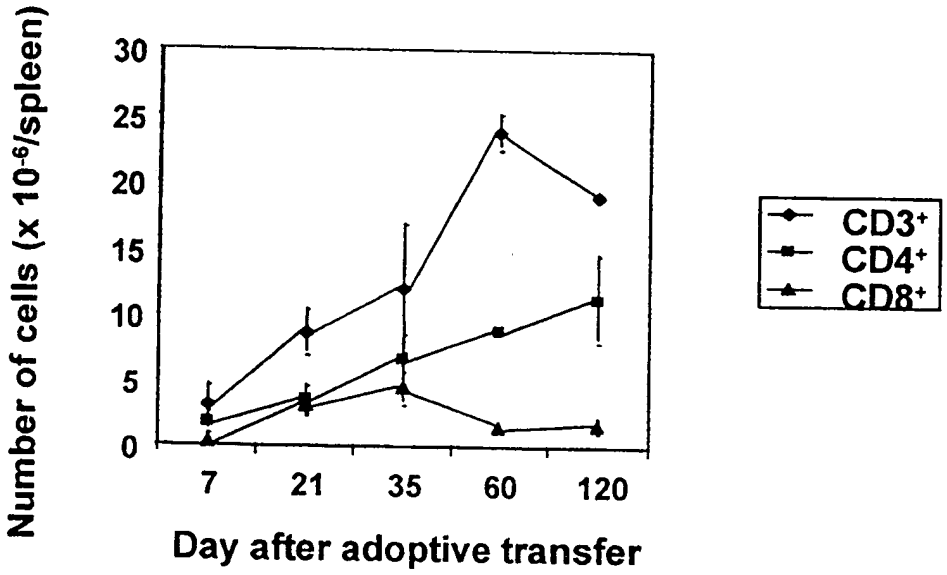


Figure 17 Cytokine Production by Spleen T Cells

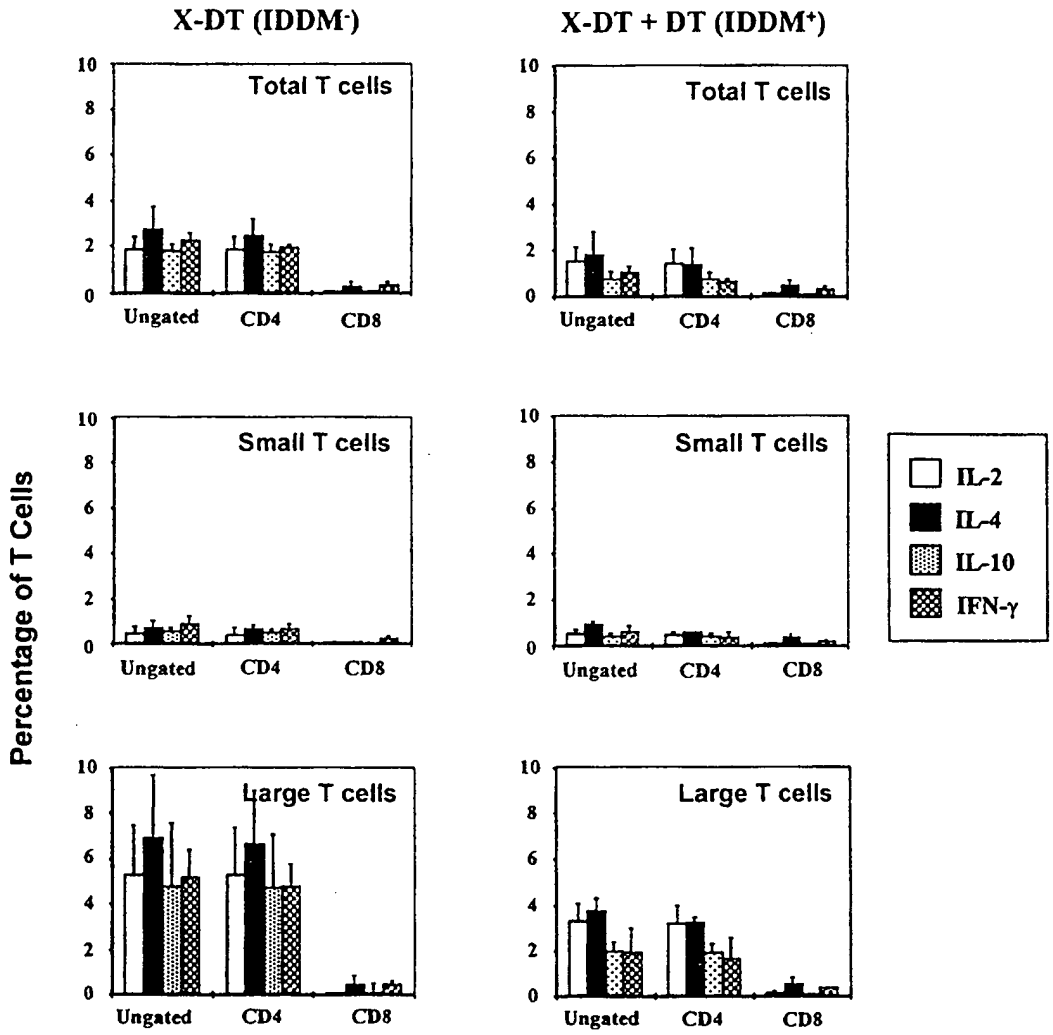


Figure 18 Cytokine Production by Mesenteric Lymph Node T Cells

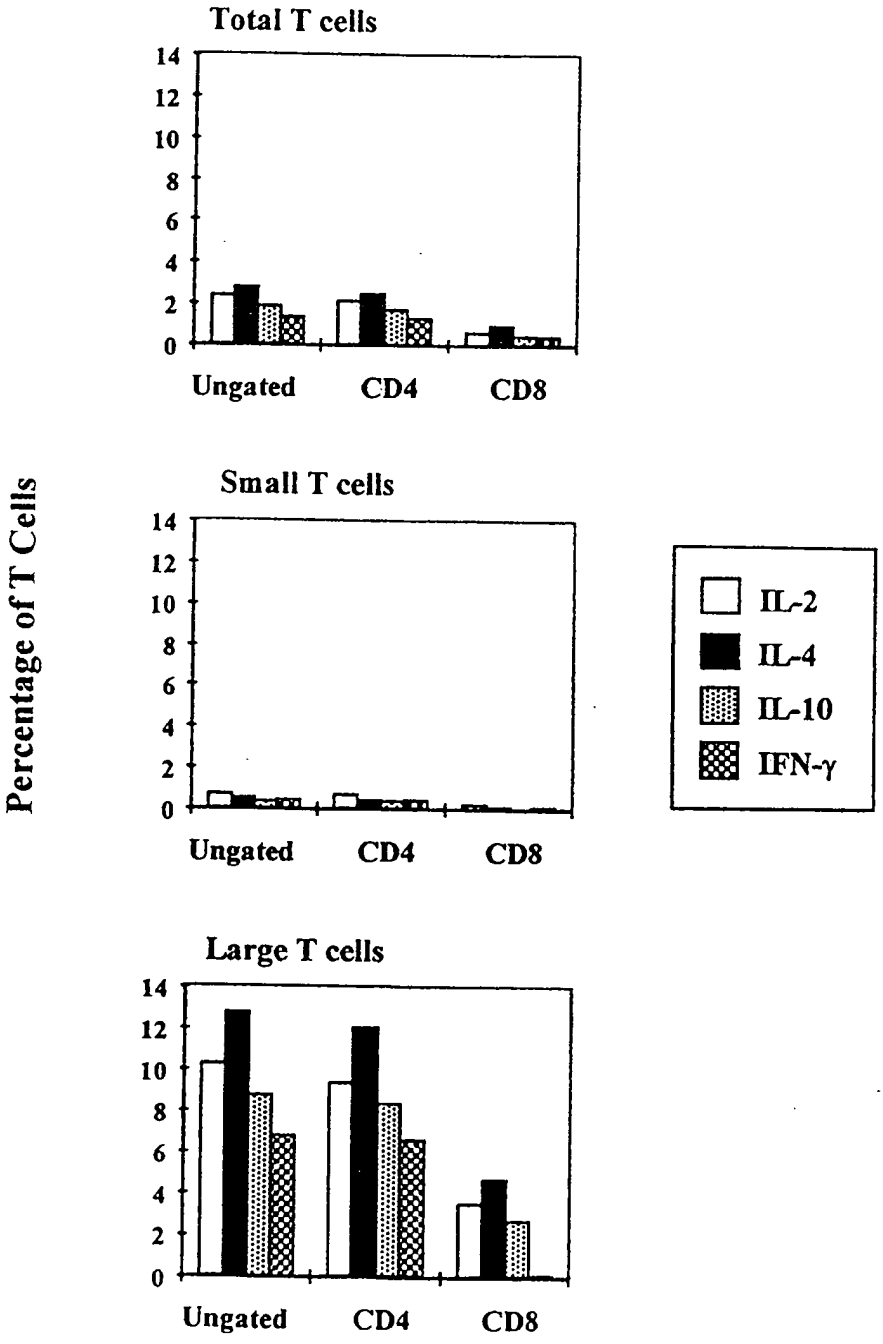


Figure 19A Resistance to DT Cell Challenge

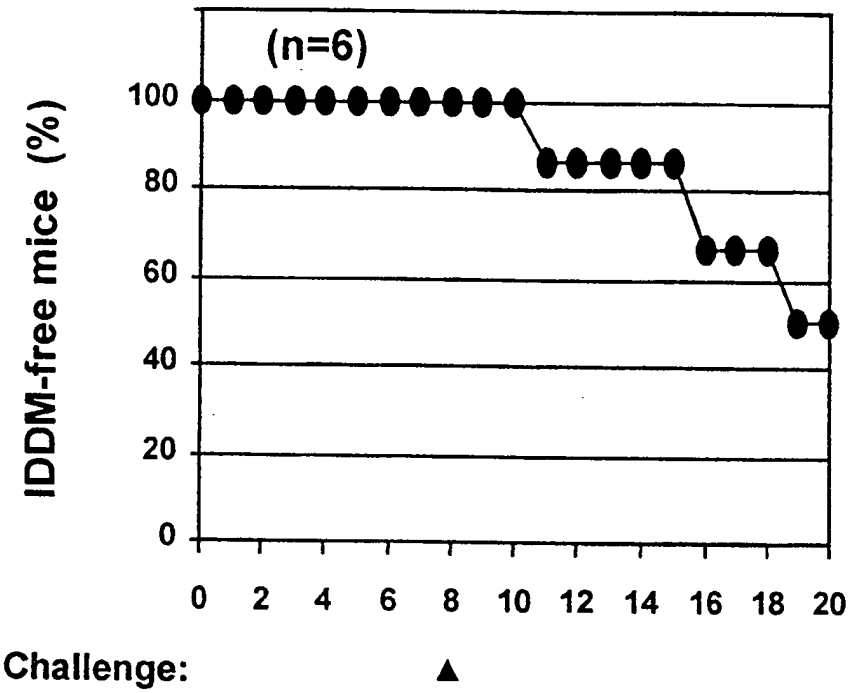


Figure 19B Repopulation of DT Cells

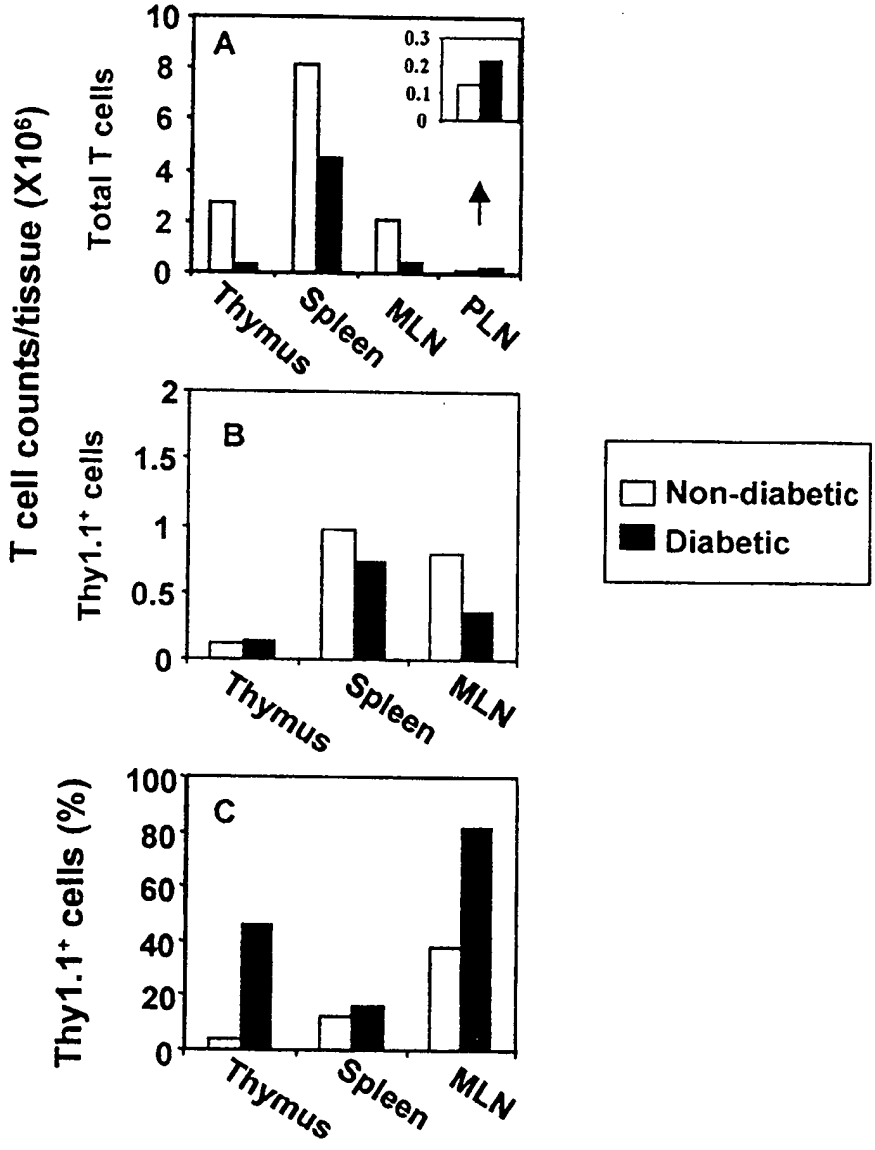


Figure 19C IL-4 Producing Cells and Diabetes

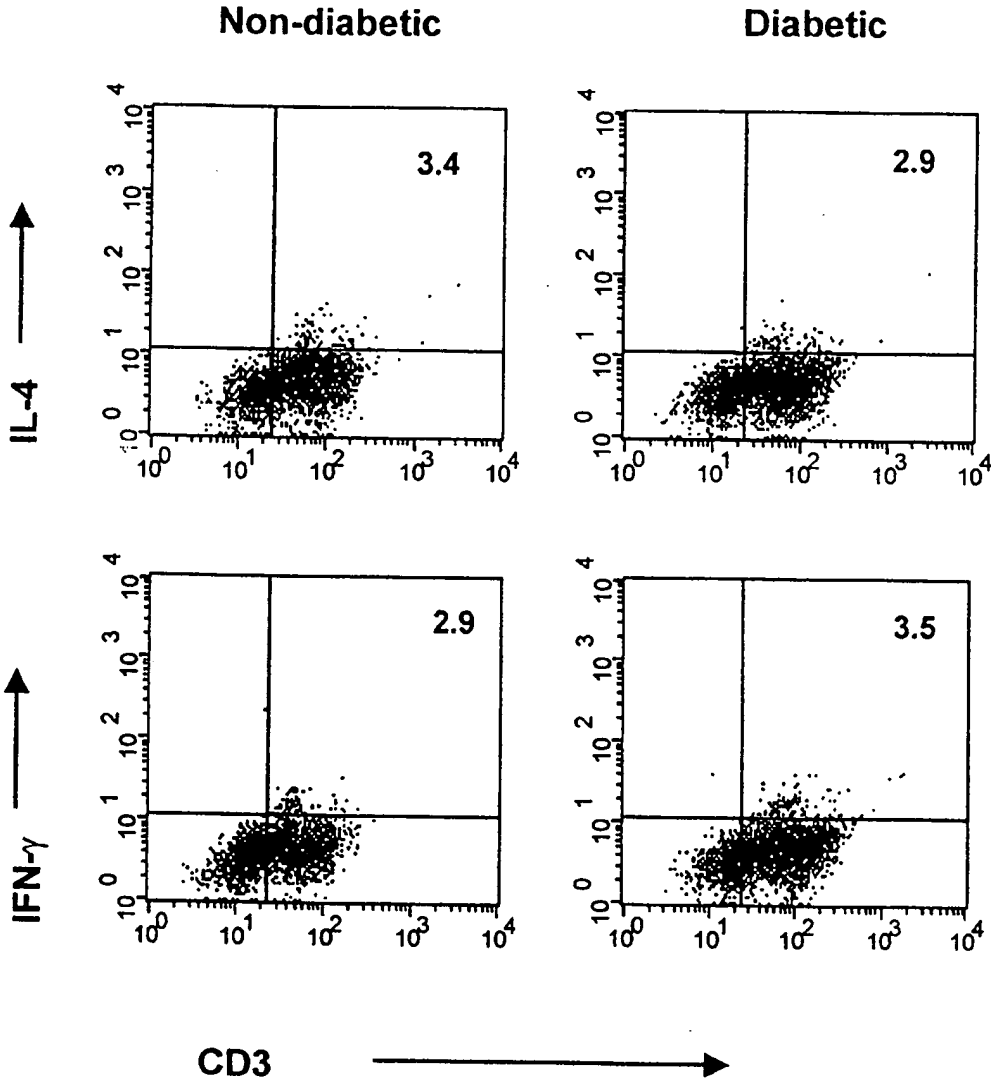


FIGURE 20 A

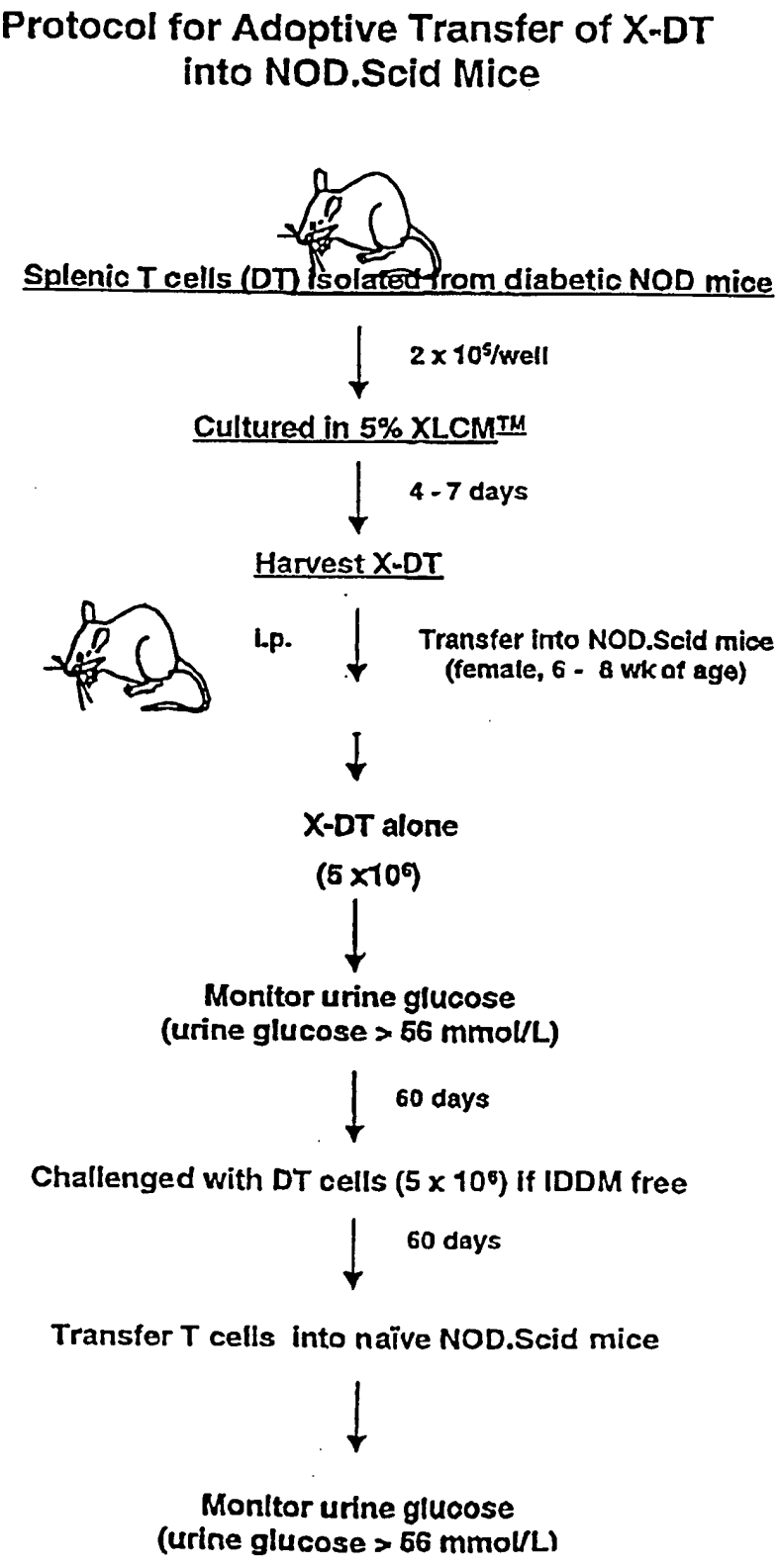

Protocol for Adoptive Transfer of X-DT into NOD.Scid Mice
Splenic T cells (DT) isolated from diabetic NOD mice
2 x 10⁶/well
Cultured in 5% XLCM™
4 - 7 days
Harvest X-DT
i.p.
Transfer into NOD.Scid mice (female, 6 - 8 wk of age)
X-DT alone (5 x10⁶)
Monitor urine glucose (urine glucose > 56 mmol/L)
60 days
Challenged with DT cells (5 x 10⁶) if IDDM free
60 days
Transfer T cells into naïve NOD.Scid mice
Monitor urine glucose (urine glucose > 56 mmol/L)

Figure 20B

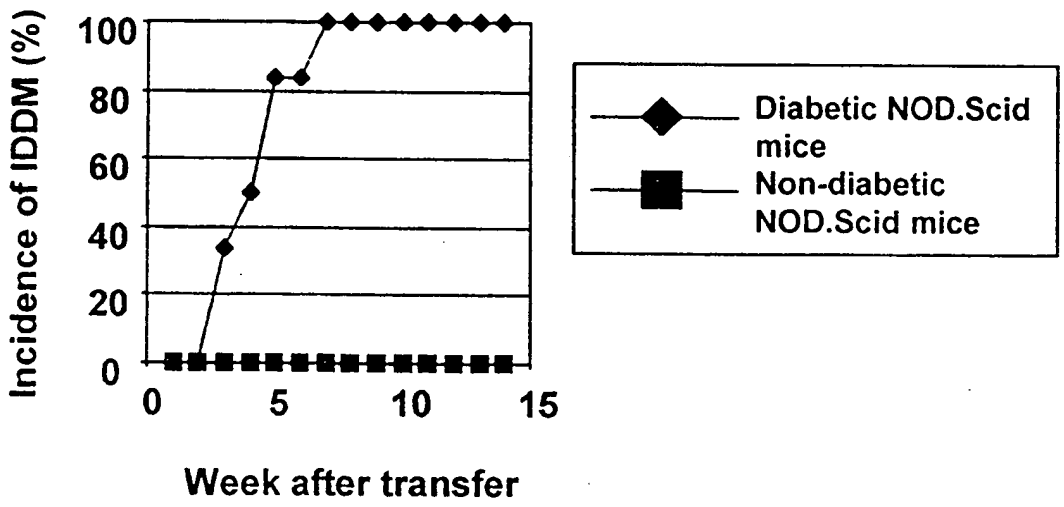

Incidence of IDDM (%)
100
80
60
40
20
0
0
5
10
15
Week after transfer
Diabetic NOD.Scid mice
Non-diabetic NOD.Scid mice

Figure 21A CD4+ X-DT Cells

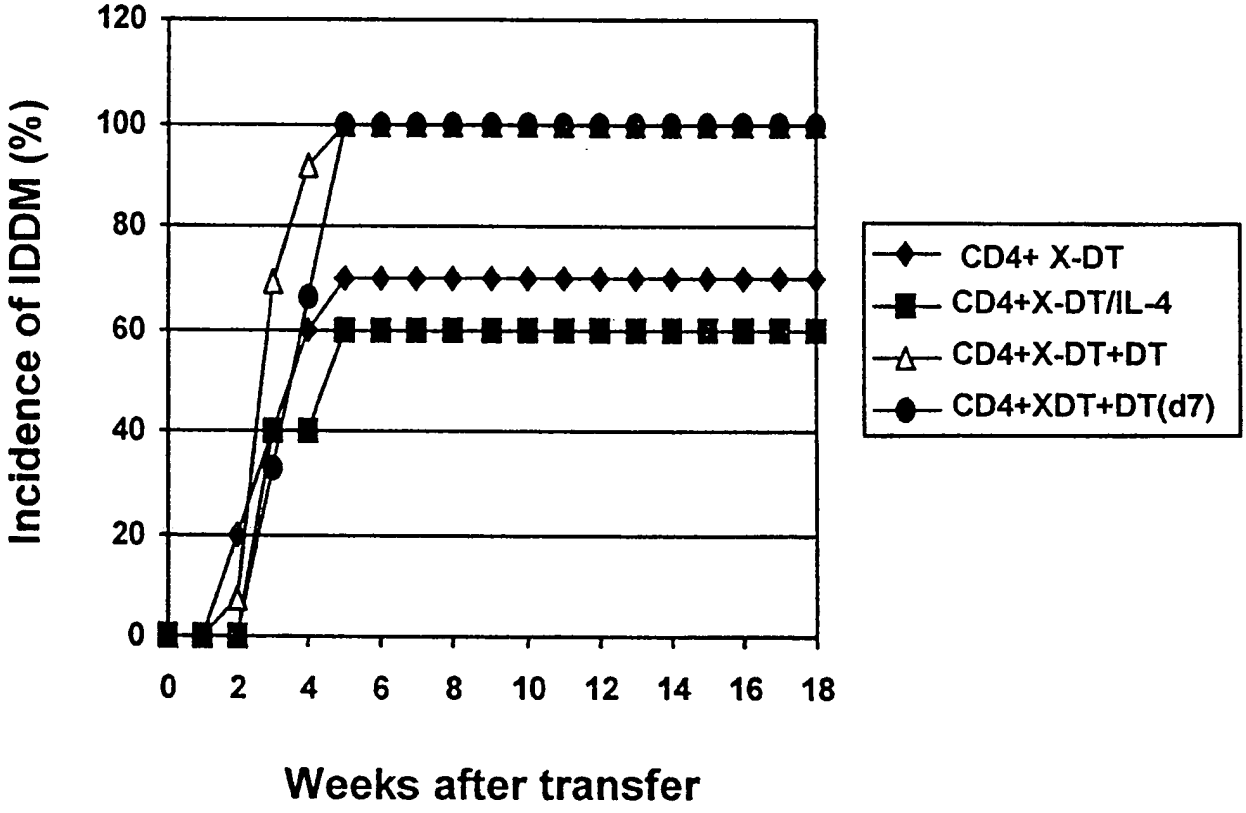
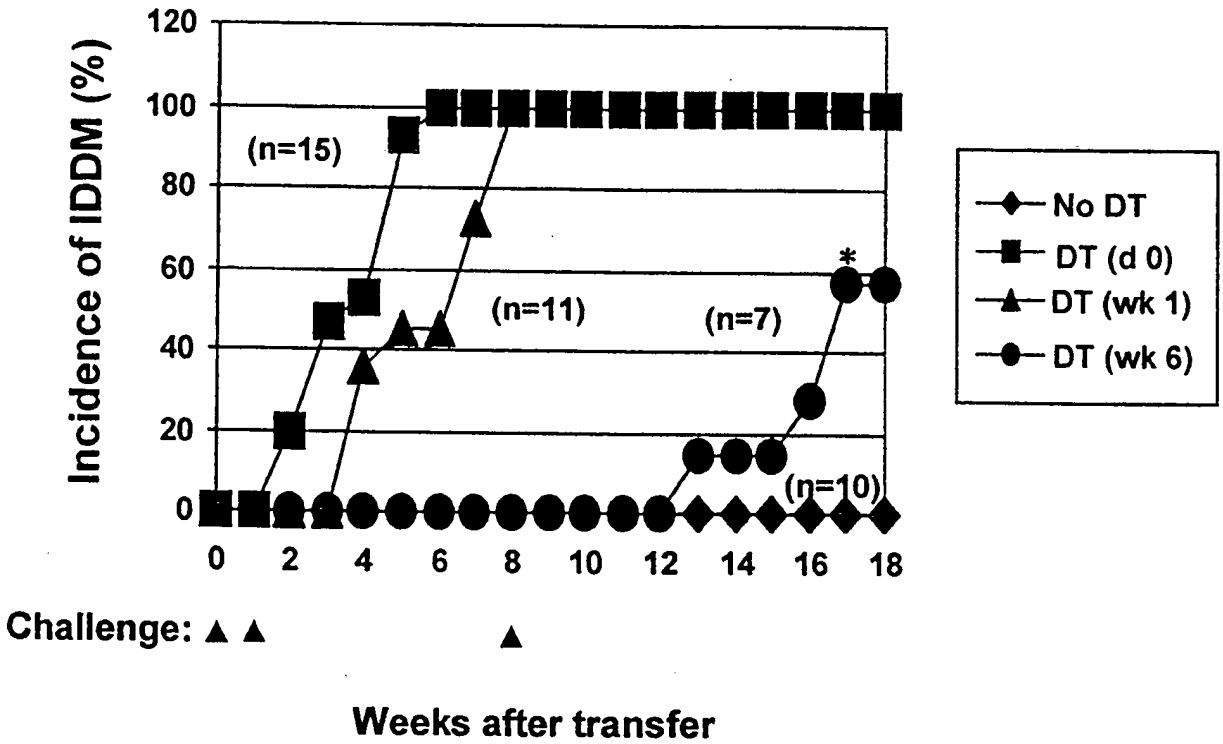


Figure 21B CD8+ X-DT Cells



INTERNATIONAL SEARCH REPORT

Inte. .ional Application No
PCT/CA 99/00069

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MINGARI M C ET AL: "Development in vitro of human CD4 + thymocytes into functionally mature Th2 cells. Exogenous interleukin-12 is required for priming thymocytes to produce both Th1 cytokines and interleukin-10." EUROPEAN JOURNAL OF IMMUNOLOGY, (1996 MAY) 26 (5) 1083-7, XP002105035 see the whole document ---	1-30
A	CAMERON M J ET AL: "IL-4 prevents insulitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function." JOURNAL OF IMMUNOLOGY, (1997 NOV 15) 159 (10) 4686-92, XP002105036 see the whole document ---	1-30

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
8 June 1999	21/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

1

INTERNATIONAL SEARCH REPORT

Inte. ..ional Application No
PCT/CA 99/00069

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SKEA, D. ET AL: "Large ex vivo expansion and reduced alloreactivity of umbilical cord blood T lymphocytes." BLOOD, (NOV. 15, 1997) VOL. 90, NO. 10 SUPPL. 1 PART 1, PP. 368A. MEETING INFO.: 39TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY SAN DIEGO, CALIFORNIA, USA DECEMBER 5-9, 1997, XP002105221 cited in the application see the whole document ---	1,2
P,X	LHOTAK, V. (1) ET AL: "Efficient ex vivo expansion of T cells from HIV-infected individuals using XLCMTM." BLOOD, (NOV. 15, 1998) VOL. 92, NO. 10 SUPPL. 1 PART 1-2, PP. 168A. MEETING INFO.: 40TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY MIAMI BEACH, FLORIDA, USA DECEMBER 4-8, 1998, XP002105037 see the whole document ---	1-13
P,X	ROBINSON, K. L. (1) ET AL: "Ex vivo expansion of umbilical cord blood (UCB) with XLCM leads to the preferential expansion, activation and maturation of CD4+ and CD8+ T lymphocytes: Development of UCB specific CTLs." BLOOD, (NOV. 15, 1998) VOL. 92, NO. 10 SUPPL. 1 PART 1-2, PP. 543A. MEETING INFO.: 40TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY MIAMI BEACH, FLORIDA, USA DECEMBER 4-8, 1998, XP002105038 see the whole document ------	1-13,18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 99/ 00069

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 18-23 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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60/080,533 3 April 1998 (03.04.98) US

(71) Applicant: OSIRIS THERAPEUTICS, INC. [US/US]; 2001 Aliceanna Street, Baltimore, MD 21231–2001 (US).

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(74) Agents: SQUIRE, William et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).

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Published
With international search report.
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(54) Title: USE OF HUMAN MESENCHYMAL STEM CELLS TO INDUCE T-CELL APOPTOSIS

(57) Abstract

The present invention provides a method for inducing antigen–specific T–cell lymphocyte elimination using human mesenchymal stem cells as antigen presenting cells which additionally express a molecule that induces T–cell apoptosis.

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USE OF HUMAN MESENCHYMAL STEM CELLS TO INDUCE T-CELL APOPTOSIS

This application is based on and claims priority of U.S. provisional application serial no. 60/080,533 filed April 3, 1998.

The present invention relates to the field of inducing death of specific T-lymphocyte cells which are deleterious to an organism. The present invention relates particularly to the area of autoimmune disease in humans.

Background of the Invention

The function of the immune system is to eliminate foreign cells which may contain pathogens, while maintaining unresponsiveness or "tolerance" against self-antigens. In a normal immune response, activation of naive T-cells requires recognition of a foreign antigenic fragment bound to a self MHC molecule and the simultaneous delivery of a co-stimulatory signal by a specialized antigen-presenting cell. T cell tolerance is achieved 1) in the thymus where thymocytes reactive for self-peptides are eliminated by clonal deletion (central tolerance), and 2) in the periphery by exposure to self-antigens under tolerogenic conditions (peripheral tolerance). Peripheral tolerance is manifested by clonal anergy, and by clonal deletion where autoreactive cells are eliminated.

Clonal deletion can also result from expression of cell death molecules on the antigen presenting cells. Classic examples of death molecules are Fas ligand (FasL) and TRAIL ligand, which ligate their receptors, Fas and DR4, respectively,

on activated T cells, inducing apoptosis of the T cells. The interaction of CD27, a member of the TNFR superfamily, and the CD27-ligand (CD70) also induces T cell apoptosis.

However, the immune system may generate a response against self-constituents, as happens in autoimmune disease. Autoimmune disease, wherein antibodies or T cells attack self proteins, may be caused by abnormal immune response. The cause may be an autoreactive T cell component, the T cells may themselves be pathogenic, or T cells may help trigger autoreactive B cells to produce antibodies to self antigens. Patients with autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease and myasthenia gravis, are either inadequately treated with existing non-selective drug therapies, or experience deleterious side effects from long-term immunosuppressive treatment.

Infusion of individuals with drugs that prevent T-cell activation can inhibit immune cell response, but these treatments result in general immune suppression, toxicity and sometimes death due to opportunistic infections. Because of the toxicity and incomplete response rate of conventional treatment of autoimmune diseases, alternative approaches are needed for patients who cannot withstand or do not respond to drug therapy.

Summary of the Invention

It has been discovered that human mesenchymal stem cells can be used to deliver antigens to the immune system for interaction with T cells. Mesenchymal stem cells can further be used to present to the immune system molecules that induce apoptotic death in cells of the immune system that express receptors for the molecules.

Accordingly, the methods of the present invention are particularly useful for eliminating, reducing or ameliorating unwanted or activated T cell responses and

can be used as a method to treat or inhibit specific unwanted or abnormal immune responses such as occurs in autoimmune disease.

In one aspect the method involves reducing, ameliorating or eliminating T cells that have been activated against an antigen by administering to a subject autologous human mesenchymal stem cells which have been modified to present such antigen, and to express a molecule that induces apoptosis of activated T cells. The mesenchymal stem cells can be used to deliver to the immune system a molecule that induces apoptosis of activated T cells since activated T cells carry a receptor for the molecule. This results in the deletion of activated T lymphocytes and in the suppression of an unwanted immune response. In accordance with an aspect of the invention, autologous human mesenchymal stem cells are modified to express a cell death molecule. In a preferred embodiment, the mesenchymal stem cells express the cell death molecule Fas ligand which will interact with the Fas receptor found on activated T cells.

Thus, the method of the present invention provides administering to a host a human mesenchymal stem cell that (i) has been modified to have at least one exogenous antigen fragment bound to a primary surface molecule of the cell such that the antigen fragment is presented to the immune system, and (ii) has been modified to express a cell death molecule. The mesenchymal stem cell presents the antigen and thereby interacts with T cells that have previously been activated. The mesenchymal stem cells of the invention further contain exogenous genetic material that codes for a molecule that induces activated T cell apoptosis. Preferably, the exogenous genetic materials are in one or more expression vectors.

In another aspect, the mesenchymal stem cells are modified to deliver to the immune system a molecule that induces activated T cell elimination. The mesenchymal stem cells, which may be allogeneic to the host, are modified to express a cell death molecule such as Fas ligand or TRAIL. When the mesenchymal stem cell comes into contact with an activated T cell, apoptosis of the activated T cell will be induced.

The mesenchymal stem cell-antigen presentation system described herein has a wide range of applications, including but not limited to, deletion of large numbers of antigen-specific T cells for use in immunotherapy against, *inter alia*, autoimmune disease.

Detailed Description of Preferred Embodiments

The invention relates to methods for reducing, inhibiting or eliminating an immune response to an antigen, *in vivo*, by employing human mesenchymal stem cells to present antigen and to simultaneously present "a cell death molecule", a molecule that induces immune cell apoptosis. The administration of these modified mesenchymal stem cells results in the deletion of activated T cells and thus a reduction of the T cell response. The human mesenchymal stem cells are preferably autologous to the recipient of the human mesenchymal stem cells.

Accordingly, the invention relates to a method of eliminating activated T cells by administering, *in vivo*, mesenchymal stem cells which deliver a specific antigen to T cells, and in addition are modified to express a cell death molecule. The present invention is based in part on the discovery that human mesenchymal stem cells do not provide costimulatory signals to fully stimulate T cells. Therefore, when antigen bearing mesenchymal stem cells are present in the immune system, the mesenchymal stem cells present the antigen without providing the costimulatory signal required for T cell activation.

In one embodiment of the invention, the mesenchymal stem cells are modified to present antigen to T cells by contacting the mesenchymal cells with antigen, *in vitro*, prior to contact with the T cells. For human mesenchymal stem cells modified to have at least one exogenous antigen fragment, the antigen can be a protein, a polypeptide, lipid or glycoprotein bound to a primary surface molecule of the cell. Thus, in this embodiment, the mesenchymal stem cell is contacted with at least one antigen (antigen-pulsing) which the mesenchymal stem cell processes into an antigen fragment.

The mesenchymal stem cells can alternatively be genetically manipulated to express an antigenic molecule. Thus, in another embodiment, the mesenchymal stem cell contains exogenous genetic material that codes for at least one exogenous antigenic polypeptide, which the mesenchymal stem cell expresses, processes into an antigen fragment and presents to the T cells.

In a preferred embodiment of this aspect of the invention, the mesenchymal stem cells are modified to present an autoantigen, for example, an autoantigen that mediates the immune response in an autoimmune disease. In accordance with this aspect, the mesenchymal stem cells are preferably autologous to the recipient of the mesenchymal stem cells. This method can be used to reduce or inhibit a T cell immune response involved in autoimmune disease, for example, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease and myasthenia gravis. By using mesenchymal stem cells that present the autoantigen against which the T cells have been activated, such activated T cells will recognize the presented antigen.

In accordance with this embodiment of the invention, the mesenchymal stem cells are also modified to express a molecule that will induce T cell apoptosis, i.e., a cell death molecule. As defined herein a "cell death molecule" is a molecule that interacts or binds with its cognate receptor on an activated T cell, the interaction inducing T cell death or apoptosis.

Fas mediates apoptosis of recently activated T cells which are again exposed to stimulation (Parijs, et al, 1996). Fas is a type I membrane receptor that, when crosslinked by its cognate ligand, induces apoptosis in a wide variety of cells. The interaction between the Fas molecule (CD95) on target cells and its ligand Fas L on activated T cells results in receptor aggregation, which transduces signals leading to apoptosis of the target cell. The Fas system has been shown to be involved in a number of cell functions *in vivo* including negative selection of thymocytes,

maintaining immune privilege sites within the body, and cytotoxic T-lymphocyte (CTL)-mediated cytotoxicity (Green and Ware, PNAS 1997).

Other members of the tumor necrosis factor receptor (TNFR) family have roles in programmed cell death: DR4 TRAIL receptor interacts with TRAIL ligand which can induce apoptosis in a variety of transformed cell lines (G. Pan SCIENCE, 1997); and the interaction of CD27 and its ligand CD70 (Prasad et al, PNAS 1997) also induces apoptosis. Whereas FasL expression is restricted to stimulated T cells and cites of immune privileges, TRAIL is detected in many normal tissues. Both TRAIL-ligand and CD70, but not Fas-ligand, are expressed on unmanipulated human mesenchymal stem cells. Activated, but not resting, T cells express the TRAIL receptor and CD27. Thus, in accordance with the present invention, the mesenchymal stem cells can be induced to express an endogenous cell death molecule or can be genetically engineered to express exogenous molecules that cause cell death.

It is believed that the mesenchymal stem cells which present an antigen to which T cells have been previously activated cause the T cells to be drawn to such mesenchymal stem cells. The activated T cells express either TRAIL-receptor, Fas or CD27 on the T cell. The engagement of these receptors with their ligands on the mesenchymal stem cells results in T cell death via apoptosis. Other ligands either present within the mesenchymal stem cell or introduced into the mesenchymal stem cell can bind to their cognate receptors on the activated T cells to induce apoptosis. In this manner, mesenchymal stem cells administered to an individual can delete autoreactive cells, reducing the severity or incidence of autoimmune disease.

An advantage of the method of the present invention over current treatment for autoimmune disease is specificity; mesenchymal stem cells can be targeted to reduce a specific immune response while reducing or eliminating the effect on other segments of the immune system. The elimination of an antigen specific immune response enables the treatment of or prevention of an unwanted or abnormal immune response to a specific antigen. The methods of the present invention are

particularly applicable to therapy of autoimmune disease and preferably eliminate the response to autoantigen specifically, while reducing or eliminating the effect on other aspects of the immune system.

The invention can be utilized for treatment of autoimmune diseases where the autoantigen mediating the disease is known. The method involves genetically engineering mesenchymal stem cells, to express an autoantigen in order to induce specific immunotherapy to inactivate or eliminate abnormal immune responses. Accordingly, the invention encompasses administering the mesenchymal stem cells to a host as a method for the treatment of autoimmune diseases such as myasthenia gravis or rheumatoid arthritis.

In another aspect of the invention, the mesenchymal stem cells are modified to express a molecule that will induce T cell apoptosis, without modification to present an antigen against which the T cells have been activated. The mesenchymal stem cells thus modified will have a nonspecific effect on the immune system, i.e., will eliminate activated T cells at or near the site of administration of the mesenchymal stem cells. Upon contact with the mesenchymal stem cells, apoptosis of the activated T cells will be induced.

Mesenchymal stem cells are the formative pluripotential blast cells found, *inter alia*, in bone marrow, blood, dermis and periosteum. These cells can be expanded in culture, for example by methods described for isolating, purifying, and greatly replicating these cells in culture, *i.e., in vitro,* in Caplan and Haynesworth, U.S. Patent No. 5,486,359.

The human mesenchymal stem cells of the invention can be engineered (transduced or transformed or transfected) with genetic material of interest. The engineered human mesenchymal stem cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying exogenous genes therein. The culture conditions, such as temperature,

pH and the like, can be those previously used with engineered human mesenchymal stem cells. See, for example, Gerson *et al.*, U.S. Patent No. 5,591,625.

Unless otherwise stated, genetic manipulations are performed as described in Sambrook *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

The mesenchymal stem cells and method of the invention can be appropriately applied to treatment strategies requiring immunosuppressive reagents. Accordingly, the present invention provides for the modification of and expansion of mesenchymal stem cells *in vitro* for use in cellular immunotherapy, and the *in vivo* administration of the immunosuppressive mesenchymal stem cells for treating or ameliorating unwanted immune responses. One aspect of the invention is the development of the mesenchymal stem cells into a vehicle for presenting antigen and delivering a cell death molecule for eliminating a specific cellular response.

The dosage of the active ingredient varies within wide limits and will, of course be fitted to the individual requirements in each particular case. In general, in the case of parenteral administration, it is customary to administer from about 0.5 to about 5 million cells per kilogram of recipient body weight. The number of cells used will depend on the weight and condition of the recipient and other variables known to those of skill in the art. The cells can be administered by a route which is suitable for the particular disease state to be treated. The antigen-modified mesenchymal stem cells can be targeted to a particular tissue or organ such as bone marrow.

The cells can be suspended in an appropriate diluent, at a concentration of from about 5×10^6 to about 50×10^6 cells/ ml. Suitable excipients for injection solutions are those that are biologically and physiologically compatible with the recipient, such as buffered saline solution. The composition for administration should be sterile, stable and physiological acceptable.

It is contemplated that the mesenchymal stem cells of the present invention can be used in conjunction with current modes of treating autoimmune disease. By ameliorating the severity of the immune response in autoimmune disease, the amount of drug used in treatment and/or the frequency of administration of drug therapy can be reduced, resulting in alleviation of general immune suppression and unwanted side effects.

What Is Claimed Is:

1. A method of eliminating T cells comprising administering to a host a human mesenchymal stem cell which expresses a cell death molecule.

2. The method of claim 1 wherein the cell death molecule is selected from the group consisting of Fas Ligand, TRAIL ligand and CD27 ligand.

3. A method of reducing T cells activated against an antigen, comprising administering to a host human mesenchymal stem cells which present the antigen against which the T cells have been activated, and which express a cell death molecule.

4. The method of claim 3 wherein the antigen is an autoantigen.

5. The method of claim 3 wherein the mesenchymal stem cells are autologous to the host.

6. The method of claim 3 wherein the cell death molecule is selected from the group consisting of Fas Ligand, TRAIL ligand and CD27 ligand.

7. Use of mesenchymal stem cells which express a cell death molecule for the preparation of a composition for eliminating T cells.

8. Use of mesenchymal stem cells which present an antigen and which express a cell death molecule for the preparation of composition for reducing T cells activated against the antigen.

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 99/05349

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRUDER S P ET AL: "Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and followin cryopreservation." JOURNAL OF CELLULAR BIOCHEMISTRY, (1997 FEB) 64 (2) 278-94. JOURNAL CODE: HNF. ISSN: 0730-2312., XP002109558 United States see the whole document ---	1-8
A	WO 95 35321 A (GSF FORSCHUNGSZENTRUM UMWELT ;THIERFELDER STEFAN (DE)) 28 December 1995 see the whole document ---	1-8
A	WO 94 03202 A (US HEALTH) 17 February 1994 see the whole document -----	1-8

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19 July 1999	13.08.99

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/05349

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-6 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the human mesenchymal stem cells.

2. [ ] Claims Nos.:
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because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

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4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intr tional Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9535321 A	28-12-1995	DE 4421391 C	30-11-1995
		AT 175215 T	15-01-1999
		CA 2193092 A	28-12-1995
		DE 59504707 D	11-02-1999
		EP 0802924 A	29-10-1997
		ES 2127531 T	16-04-1999
		JP 10505325 T	26-05-1998
		US 5830473 A	03-11-1998
WO 9403202 A	17-02-1994	AT 151989 T	15-05-1997
		AU 688736 B	19-03-1998
		AU 4805293 A	03-03-1994
		CA 2140878 A	17-02-1994
		DE 69310182 D	28-05-1997
		DE 69310182 T	04-12-1997
		EP 0655927 A	07-06-1995
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(57) Abstract

Splice variants of known TAP1 and TAP2 proteins, which are involved in translocation of antigen peptides into the endoplasmic reticulum for complexing with MHC class I molecules and eventual display on the cell surface, are disclosed. Two fully sequenced and characterized splice variants of TAP1 and TAP2, designated TAP1iso[3] and TAP2iso, respectively are disclosed. The TAP2iso protein subunit is shown to form functional heterodimers with TAP1 and to exhibit a peptide specificity that differs from previously studied TAP1/TAP2 transporter proteins. The discovery of splice variant TAP subunits alters the prior theory of immune response and introduces a cellular mechanism for diversification of antigen display to the CD8–positive T cells of the immune system. method for diagnosis and treatment of diseases or conditions associated with abnormal TAP isoform expression, or of expanding the repertoire of antigen peptides to which an individual's immune system is capable of responding, are also disclosed.

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TRANSPORTER PROTEIN SPLICE VARIANTS AND MODEL FOR IMMUNE DIVERSITY

Field of the Invention

The present invention relates to the discovery of nonallelic TAP polymorphs and to a new model of immune diversity based by this discovery. More specifically, the invention relates to TAP splice variants, which lead to the formation of a previously unknown class of MHC class I antigen complexes being presented to the immune system on MHC class I-presenting cells. A new layer of diversity is thus discovered for the immune system, in addition to the allelic variation in MHC class I molecules and the genetic rearrangements leading to diversity in the T cell receptor repertoire.

Background of the Invention

Class II and class I proteins encoded by genes of the Major Histocompatability Complex (MHC) on chromosome 6 in humans play an essential role in regulating the immune system. MHC class II molecules, which are expressed in antigen-presenting cells such as macrophages, B cells, monocytes and some epithelial cells, form complexes with antigen peptides ("MHC class II antigen complexes") that are displayed on the surface of antigen-presenting cells for recognition by CD4+ T lymphocytes (helper T cells). Helper T cell recognition results in release of lymphokines and T-dependent activation of B cells, which, in turn, lead to activation of macrophages and release of

antibodies from B cells, leading to the killing or elimination of invading microorganisms. MHC class I molecules, which are expressed in virtually all nucleated cells, form complexes with antigen peptides ("MHC class I antigen complexes") that are displayed on the cell surface for recognition by CD8+ cytotoxic T lymphocytes (CTLs). Presentation of an endogenous or "self" peptide by the MHC class I antigen complex is protective, the CTLs that would otherwise recognize the surface complex and attack the presenting cell (i.e., autoreactive CTLs) having been eliminated (deleted) from the immune system repertoire; and presentation of an exogenous (foreign or "non-self") peptide (or a mutated endogenous peptide) by the MHC class I antigen complex elicits CTL attack and cytolytic destruction of the infected or diseased cell.

The peptides that complex with MHC molecules are approximately eight to twenty-four amino acids in length. In the case of class II antigen complexes, the peptides are derived from partial proteolysis and processing of extracellular antigenic proteins incorporated by the cell through phagocytosis or pinocytosis or possibly surface processing. Thus, the immune recognition events mediated by MHC class II antigen complexes are a primary defense to invading microorganisms (e.g., bacteria, parasites) or foreign substances (e.g., haptens, transplant tissues) introduced to the cells of the immune system via the circulatory or lymph systems. In the case of class I antigen complexes, the antigen peptides are derived from intracellular processing of proteins. Thus, MHC class I antigen complexes either mark the cell as a normal endogenous cell, which elicits no immune response, or mark the cell as an infected cell (e.g., as in the case of a virus-infected cell exhibiting intracellularly processed viral (i.e., foreign) peptide in the surface MHC class I complex) or a transformed cell (e.g., such as a malignant cell), which marks the cell for attack by CTLs.

Proper intracellular processing of antigen peptides for MHC class I complexing and presentation involves several steps. One of these steps is transport of the peptides from the cytosol into the endoplasmic reticulum (ER), where coupling of the antigen peptide with the MHC class I molecule takes place. The MHC class I antigen complex migrates to the cell surface for presentation and possible recognition by T cells. Unsuccessful transport of peptides into the ER, or other abnormalities leading to faulty class I antigen complex formation or presentation, can lead to a failure in recognizing autologous cells as "self". For example, defects in genes coding for transporter proteins have been discovered to be an underlying cause of several autoimmune diseases (Faustman et al., Science, 254:1756-1761 (1991); U.S. Patent No. 5,538,854).

Transporter associated with Antigen Processing, or TAP, proteins transport peptide fragments of eight or more amino acids from the cytosol of a cell into the lumen of the ER, where the peptides are bound by MHC class I proteins to form an antigen complex, which ultimately is displayed on the surface of the cell (see, e.g., Powis et al., Immunogenetics, 37:373-380 (1990)).

The TAP protein is a heterodimer of the products of the *TAP1* and *TAP2* genes, which are also located in the MHC region of the genome. Each subunit of the TAP1/TAP2 heterodimer forms an ATP-binding domain and a domain that criss-crosses the membrane six to eight times, and both subunits are required to form a peptide binding site and to translocate peptide into the ER (Androlewicz et al., Proc. Natl. Acad. Sci. USA, 91(26): 12716-12720 (1994); Hill et al., Proc. Natl. Acad. Sci. USA, 92: 341-343 (1995)).

The role of TAP in mediating the supply of antigen peptides transported into the ER and ultimately displayed by MHC class I molecules has caused close scrutiny of the range of peptides capable of translocation by TAP, to determine whether TAP is a further restrictive factor in immune diversity. (See, Hill et al., *ibid.*; Howard, Proc. Natl. Acad. Sci. USA, 90: 3777-3779 (1993).) Whereas gene rearrangement during ontogeny of T cells generates an enormous variety of T cell receptor specificities, making the recognition capability of the immune system very diverse, there has not been discovered any corresponding mechanism for diversifying the presentation capability of an individual's immune system. Although small variations in MHC allotypes result in different repertoires of antigen peptides being complexed and presented by MHC molecules, lending diversity to antigen presentation across a species, an individual's MHC haplotypes restrict the range of antigens that can be effectively displayed. The specificity of the TAP transport mechanism also shapes the repertoire of antigen complexes presented to the immune system, in that only peptides capable of translocation by TAP are made available for complexing in the ER with MHC class I. (See, Howard, *ibid.*)

The peptide specificity of TAP proteins has been studied in three species thus far: human, mouse and rat. In the rat, it was shown that different alleles of the *TAP2* gene gave rise to functional polymorphism, i.e., the different alleles transported sets of peptides that differed in C-terminal residues. (Powis et al., Immunity, 4(2):159-165 (1996); Powis et al., Nature, 357:211-215 (1992).) In the human and mouse, however, investigation of several polymorphs of TAP1 and TAP2 did not reveal any alteration in the spectrum of peptides transported, and it has been generally concluded that although in mice and humans the TAP1 and TAP2 proteins are genetically polymorphic, they are functionally monomorphic, the sequence alterations of the allotypes causing no shift in the types of peptides translocated by TAP. (Schumacher et al., Proc. Natl. Acad. Sci. USA, 91(26):13004-13008 (1994); Obst et al., Eur. J. Immunol., 25: 2170-2176 (1995); Daniel et al., J. Immunol., 159: 2350-2357 (1997).)

It has now been unexpectedly discovered that the human *TAP1* and *TAP2* genes produce several splice variants that differ structurally and functionally from the known TAP1 and TAP2

proteins, and functional TAP1 and TAP2 splice variant gene products, designated TAP1iso[3] and TAP2iso, have been characterized and their full coding sequences isolated.

The TAP heterodimer including the TAP2iso splice variant, surprisingly, preferentially translocates a different set of peptides than the TAP heterodimer including TAP2. These discoveries have led to a revision described herein of the model of peptide transport into the lumen of the ER for MHC class I complexing; and a new level of diversity in the presentation of antigen complexes, akin in some respects to the diversity of T cell receptors in the recognition of such complexes, has been exposed for the first time.

Summary of the Invention

The present invention provides novel TAP1 and TAP2 splice variants and novel functional TAP heterodimers (i.e., a TAP1 subunit complexed with a TAP2 subunit) including at least one splice variant of TAP1 or TAP2. The TAP splice variants alter peptide transport to the ER in comparison with the previously known TAP complex. "Altered peptide transport" includes preferential transport of a specific repertoire of peptides that differs (e.g., in length or amino acid content or structure) from that transported by the previously known TAP protein (TAP1/TAP2 heterodimer), and/or a change in the rate of peptide transport into the ER as compared to known TAP. TAP splice variants according to the invention preferably measurably affect the repertoire of peptides displayed as MHC class I antigen complexes on the surface of antigen presenting cells or affect the cell surface density of particular MHC class I antigen complexes.

A specific embodiment of the present invention is designated TAP2iso (SEQ ID NO: 2). TAP2iso differs from the previously known TAP2 protein (see SEQ ID NO: 15) by a deletion of a C-terminal sequence corresponding to exon 11 of the human gene encoding TAP2 and the insertion in-frame with exon 10 of newly discovered exon 12 (SEQ ID NO: 5). Isolated nucleic acids encoding TAP2iso are also disclosed (see, e.g., TAP2iso cDNA, SEQ ID NO: 4). Another specific embodiment is designated TAP1iso[3] (SEQ ID NO: 25), which differs from the previously known TAP1 (see SEQ ID NO: 19) by deletion of the C-terminal sequence corresponding to exons 9-11 and the transcription of additional sequence 3' of exon 8, leading to the addition of several amino acids (see item 8a in Fig. 11). Further embodiments of this invention relate to additional TAP1 and TAP2 splice variants that have been detected by RT-PCR studies. Specific additional splice variants thus far detected are designated TAP1iso, TAP1iso[2] and TAP2iso[2].

The TAP1 and TAP2 splice variants of the present invention are unique arrangements and/or combinations of TAP1 and TAP2 exons. The TAP 1 and 2 splice variants are functional in that each splice variant complexes with at least one corresponding TAP subunit to form a TAP1-type

subunit/TAP2-type subunit complex (a TAP heterodimer), which preferably is capable of translocating peptides.

The present invention relates also to novel TAP1 and TAP2 exons, by which the splice variants differ from known TAP1 and TAP2 coding sequences (see SEQ ID NOs: 18 and 17, respectively), and to the peptides encoded by those exons, which are useful, e.g., as immunogens for production of antibodies that can selectively recognize the splice variant proteins from mixed populations of TAP gene products.

The discovery of a genetic link to the repertoire of antigen peptides that are transported intracellularly to form MHC class I antigen complexes raises the possibility that variation in the complement of TAP1 or TAP2 gene products in humans will be associated with autoimmune disease. Thus, a deletion or a mutation in a splice variant exon may result in a failure to present certain endogenous peptides, leading in turn to autoreactive CTL attack of self tissues. Alteration of the ratio of co-expressed TAP subunit variants may also be associated with disease. And defective production of a particular variant of the TAP genes may provide an opening in the immune presentation-and-recognition system allowing malignant cells or virally infected cells to escape detection and to survive where they would be attacked and eliminated in another (normal) individual. This invention provides methods for detection and treatment of such disorders associated with abnormalities in TAP splice variant expression.

This invention also provides new methods for designing vaccines to broaden the immune response of individuals that may be unresponsive to a standard vaccine due to inadequate processing of particular viral antigens, for instance due to their expression of MHC class I alleles that do not efficiently display antigen peptides from available vaccine preparations. To broaden an individual's immune response to a particular virus, cells from the individual, e.g., lymphocytes (preferably macrophages, B cells or dendritic cells) are withdrawn and transfected with a gene encoding a TAP isoform that is not expressed or poorly expressed in that individual, transfectants are recovered that express the inserted TAP isoform DNA, and then the cells are returned to the individual. The TAP isoform gene may be non-specific, provided simply to broaden the range of peptides processed by the cells, or specific for providing translocation of particular viral antigen peptides. The transfected cells, expressing an additional TAP isoform will supplement the repertoire of antigen peptides processed and displayed to the immune system as MHC class I antigen complexes, leading to recognition by CD8-positive (CD8+) T cells and the development of T cell memory for those antigen complexes. Thereafter, the vaccinated individual will display a secondary antigen response to challenge from the virus. Similar methods will be useful for improving immune response against infectious diseases or tumor cells that escape an individual's normal antigen processing capability.

Brief Description of the Drawings

Fig. 1 depicts the organization of genomic human *TAP2* genes and the structures of *TAP2* and *TAP2iso* cDNAs. The open boxes represent exons 1 to 10, which, sharing the same 5' untranslated region (5'-UT), are present in both *TAP2* and *TAP2iso* cDNAs. The alternatively spliced exons 11 and 12, together with their corresponding 3' untranslated regions (3'-UT), are hatched.

Fig. 2 illustrates the sequence analysis of the exon junctions of *TAP2* (clone 0121) and *TAP2iso* (clone 01023) cDNAs isolated from the same human spleen cDNA library. Two full-length cDNA clones, 0121 and 0123, represent two different forms of human *TAP2* cDNA. Clone 0121 corresponds to the previously known TAP2 isoform, in which exon 10 is spliced at transcription to exon 11. Clone 0123 represents the newly discovered TAP2iso cDNA, in which exon 10 is spliced to exon 12.

Fig. 3 illustrates electrophoresis results of RT-PCR products showing co-expression of TAP2 and TAP2iso mRNAs in various human cell lines, along with β_2-microglobulin (β2). Lanes: (0) DNA molecular size markers; (1) T1 cells (human lymphoblastoid B cell line); (2) T2 cells (human lymphoblastoid B cell line of T1 origin with a large homozygous deletion of the MHC class II gene region containing *TAP1* and *TAP2*); (3 and 4) fresh human peripheral blood lymphocytes from two different donors; (5 and 6) Epstein-Barr virus-immortalized B cell lines from two different donors; (7) MOLT-4 acute lymphoblastic leukemia cells; (8) THP-1 monocytic cells; (9) U-937 histocytic lymphoma cells; (10) HeLa epithelioid carcinoma cells; (11) PACA pancreatic carcinoma cells.

Figs. 4A and 4B illustrate electrophoresis results showing the effect of TAP2iso together with TAP1 on the maturation of MHC class I molecules in the ER of T2 cells transfected with *TAP1* and *TAP2iso* (Fig. 4A) as compared with untransfected T2 cells (Fig. 4B) incubated in the absence (-) or presence (+) of endoglyosidase H (endo H) at various time points. "r" and "s" indicate class I proteins resistant and sensitive to endo H, respectively. The increased rate of class I maturation in *TAP1/TAP2iso*-transfected cells is reflected by the difference in endo H sensitivity compared with untransfected cells.

Fig. 5 illustrates peptide selectivity of TAP1/TAP2 and TAP1/TAP2iso heterodimers in permeabilized T2 transfectants. Three different glycosylated and radiolabeled (^{125}I) peptides, RRYQNSTEL (SEQ ID NO: 6), IYLGPFSPNVTL (SEQ ID NO: 7) and TVDNKTRYE (SEQ ID NO: 8) were recovered and measured. The data presented are means of three separate experiments employing ten different sets of transfectants. The amount of translocated peptide is shown as a percentage of the total amount of radiolabeled peptide introduced. The results demonstrate that

RRYQNSTEL was translocated with equal efficiency by TAP1/TAP2 or TAP1/TAP2iso heterodimers, but that IYLGPFSPNVTL was transported four times more efficiently by the TAP1/TAP2iso heterodimer compared to the TAP1/TAP2 transporter, and that TVDNKTRYE was transported more efficiently in the TAP1/TAP2 transfectant.

Figs. 6A, 6B, 6C, 6D and 6E illustrate competitive transport efficiency in transfected T2 cells. The peptide RRYQNSTEL (SEQ ID NO: 6) is analyzed for transport efficiency in competition with itself (Fig. 6A), in competition with IYLGPFSPNVTL (SEQ ID NO: 7) (Fig. 6B), in competition with TVDNKTRYE (SEQ ID NO: 8) (Fig. 6C), in competition with SYSMEHGRWGKPVGKKRRPVKVYP (SEQ ID NO: 9) (Fig. 6D), and in competition with RGFFYTPKA (SEQ ID NO: 10).

Fig. 7 compares relative fifty percent inhibitory concentrations (IC 50) of the test peptides (RRYQNSTEL, IYLGPFSPNVTL, and TVDNKTRYE) in T1 cells (which naturally co-express both TAP2 and TAP2iso) and T2 transfectants expressing either TAP1/TAP2 or TAP1/TAP2iso. The T1 cells show transportation of all the test peptides with equal efficiency, whereas the transfectants exhibit preferential transport of one of the three test peptides in comparison to RRYQNSTEL.

Fig. 8 illustrates electrophoresis results showing co-transcription of TAP1iso and TAP1iso^2 mRNAs in EBV-stabilized cells from various human subjects. Lanes: (1) DNA molecular size markers; (2) genomic DNA from a first individual (Control #1); (3) nuclear RNA from Control #1; (4) cytoplasmic mRNA from Control #1; (5) total RNA from Control #1, without DNAase digestion; (6) total RNA from Control #1, with DNAase digestion; (7) cytoplasmic mRNA from a second individual (Control #2), with DNAase digestion; (8) cytoplasmic mRNA from a third individual (Control #3), with DNAase digestion; (9) cytoplasmic mRNA from a fourth individual (Control #4), with DNAase digestion; (10) cytoplasmic mRNA from a diabetic patient, with DNAase digestion.

Figs. 9A and 9B show the comparative sequences of the PCR products indicating the presence of splice variants TAP1iso and TAP1iso^2 (Fig. 9A) and a schematic diagram of the genomic DNA including TAP1 exons 9-11, with diagrams of the TAP1iso and TAP1iso^2 messages, both including exons 9 and 10 but two different segments of intron 10 (Fig. 9B).

Fig. 10 illustrates electrophoresis results showing co-transcription in several individuals of a fully spliced TAP2 and a variant in some individuals presumably retaining part of intron 10. The second product indicates a further TAP2 splice variant, designated TAP2iso^2. All lanes show PCR products of cDNA derived from cytoplasmic mRNA from fresh peripheral blood lymphocytes (PBLs) from several normal and one hypothyroid individual. Lanes: (1) Control (normal) individual 1; (2) Control individual 2; (3) hypothyroid patient; (4) Control individual 3; (5) Control individual

4; (6) Control individual 5; (7) Control individual 6; (9) Control individual 7; (10) Control individual 8; (11) control genomic DNA sample.

Fig. 11 depicts the organization of genomic human *TAP1* genes and the structures of *TAP1* and *TAP1iso*[3] cDNAs. The open boxes represent exons 1 to 8, which, sharing the same 5' untranslated region (5'-UT), are present in both *TAP1* and *TAP1iso*[3] cDNAs. The exons 9, 10 and 11, together with their corresponding 3' untranslated regions (3'-UT), are hatched. These exons are spliced-in in the expression of TAP1 but are spliced-out in the discovered TAP1iso[3], which is composed of the exons 1 through 8 and additional amino acids encoded by a portion of intron 8 (marked 8a) and its corresponding 3' untranslated region (3'-UT), which are reverse-hatched.

Definitions

The following illustrative explanations are provided to facilitate understanding of certain terms and phrases frequently used and of particular significance herein. Throughout this specification, TAP proteins will be referred to using unitalicized type (e.g., "TAP2" and "TAP2iso"), and the corresponding genetic materials or polynucleotides coding for such TAP proteins will be referred to using italics (e.g., "*TAP2*" and "*TAP2iso*" genes)

The term "exon polypeptide", as used herein, refers to the peptide corresponding to the portion of a protein encoded by the DNA of a particular exon. Thus, the exon 12 polypeptide of the present invention, Lys-Thr-Leu-Trp-Lys-Phe-Met-Ile-Phe (SEQ ID NO: 1), is the polypeptide comprising the amino acids encoded by exon 12 of the *TAP2iso* coding sequence.

The term "splice variant", as used herein, refers to a gene product that is homologous to a known gene product and is generated by alternative RNA splicing during transcription. The splice variant will be partially identical in sequence to the known homologous gene product, corresponding to the extent of identity of exon use, comparing the mRNA transcripts (or cDNA), between the known product and the splice variant. Thus, the TAP2 splice variant described herein that is designated TAP2iso (SEQ ID NO: 2), is homologous to the previously known TAP2 protein (SEQ ID NO: 15), and the two gene products have common N-terminal amino acid sequences corresponding to the amino acids encoded by exons 1-10 of the *TAP2* gene, and the amino acid sequences differ C-terminally to the exon 10 polypeptide, with TAP2 terminating with the exon 11 polypeptide (SEQ ID NO: 16) but TAP2iso terminating instead with the exon 12 polypeptide (SEQ ID NO: 1). By virtue of the partial identity and partial divergence of their amino acid sequences, the splice variant and the known homologues will have some functionality in common but will differ in other functions. For example, as shown herein, TAP2 and TAP2iso both (in a TAP complex with a TAP1 subunit) perform the function of translocating peptides into the lumen of the ER for MHC

class I antigen complex assembly, but TAP2iso transports a repertoire of peptides that differs from that of TAP2, or transports the same peptides at a rate or with an efficiency that differs from TAP2.

The splice variants described herein form heterodimers with corresponding TAP subunits, and the functional TAP proteins formed by association of a splice variant with a known TAP subunit or by association of two splice variants are also new. TAP heterodimers including at least one subunit that is a splice variant will be referred to herein as "TAPiso" proteins, which will be understood to be comprised of one TAP1-type subunit and one TAP2-type subunit, where one or both of the subunits are splice variants of the known TAP1 and TAP2 subunits (e.g., TAP1iso/TAP2, TAP1/TAP2iso, TAP1/TAP2iso[2] and TAP1iso/TAP2iso heterodimers, and the like, are specific examples of "TAPiso proteins").

The term "homologous", as used herein, refers to the degree of sequence similarity between two polymers (i.e., polypeptide molecules or nucleic acid molecules). When the same nucleotide or amino acid residue occupies a sequence position in the two polymers under comparison, then the polymers are homologous at that position. The percent homology between two polymers is the mathematical relationship of the number of homologous positions shared by the two polymers divided by the total number of positions compared, the product multiplied by 100. For example, if the amino acid residues at 60 of 100 amino acid positions in two polypeptide sequences match or are homologous then the two sequences are 60% homologous. The homology percentage figures referred to herein reflect the maximal homology possible between the two polymers, i.e., the percent homology when the two polymers are so aligned as to have the greatest number of matched (homologous) positions.

The present invention further relates to isolated nucleic acids (or "polynucleotides") that hybridize to the TAP splice variant nucleic acid sequences described herein if there is sufficient homology between the TAP coding sequence and the complement of the homologous coding sequence to hybridize to each other under conditions equivalent to, e.g., about 20° to 27°C below T_m and 1M NaCl. The present invention particularly contemplates nucleic acid sequences that hybridize under stringent conditions to the TAP splice variant coding sequences described herein and complementary sequences thereof. For the purposes of this invention, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the nucleic acid sequences. Thus, the present invention particularly contemplates polynucleotides encoding TAP splice variants having the particular nucleic acid sequences described herein, or polynucleotides that are at least 95% identical to such sequences, and polynucleotides having sequences that are complementary to the aforementioned polynucleotides. The polynucleotides of the present invention that hybridize to the complement of TAP splice variant

coding sequences described herein preferably encode polypeptides that retain substantially the same biological function or activity as the mature TAP splice variant polypeptides encoded by the splice variant sequences to which they hybridize.

The term "isolated" means that the material is removed from its original or native environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated by human intervention from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of the environment in which it is found in Nature. Similarly, as used herein, the term "substantially purified" is used in reference to a substance that has been separated or otherwise removed, through human intervention, from the immediate chemical environment in which it occurs in Nature. Substantially purified polypeptides or nucleic acids may be obtained or produced by any of a number of techniques and procedures generally known in the field.

The present invention incorporates by reference methods and techniques well known in the field of molecular and cellular biology. These techniques include, but are not limited to techniques described in the following publications:

Old, R.W. & S.B. Primrose, Principles of Gene Manipulation: An Introduction To Genetic Engineering (3d Ed. 1985) Blackwell Scientific Publications, Boston. Studies in Microbiology; V.2:409 pp. (ISBN 0-632-01318-4).

Miller, J.H. & M.P. Calos eds., Gene Transfer Vectors For Mammalian Cells (1987) Cold Spring Harbor Laboratory Press, NY. 169 pp. (ISBN 0-87969-198-0).

Mayer, R.J. & J.H. Walker eds., Immunochemical Methods In Cell and Molecular Biology (1987) Academic Press, London. 325 pp. (ISBN 0-12480-855-7).

Sambrook, J. et al. eds., Molecular Cloning: A Laboratory Manual (2d Ed. 1989) Cold Spring Harbor Laboratory Press, NY. Vols. 1-3. (ISBN 0-87969-309-6).

Winnacker, E.L., From Genes To Clones: Introduction To Gene Technology (1987) VCH Publishers, NY (translated by Horst Ibelgaufts). 634 pp. (ISBN 0-89573-614-4).

Description of the Preferred Embodiments

The present invention relates to the discovery of previously unknown isoforms homologous to the known TAP protein subunits (TAP1 and TAP2, see SEQ ID NOs: 18 and 17 (coding sequences) and SEQ ID NOs: 19 and 15 (amino acid sequences)). The newly discovered isoforms are the result of alternate RNA splicing and are co-expressed with the known TAP1 and TAP2 gene products, providing a plurality of TAP heterodimers functioning to translocate antigen peptides from the cytoplasm into the endoplasmic reticulum for complexing with MHC class I molecules and formation of MHC class I antigen complexes. The splice variant isoforms have been found to form TAP heterodimers that transport a different repertoire of peptides or that transport similar peptides at different rates (i.e., with greater or lesser efficiency) than the known TAP1/TAP2 heterodimer; and the discovery of these alternate TAP transporter proteins exposes a genetic mechanism of diversification in the process of MHC class I antigen presentation. Co-expression of multiple TAP1 and TAP2 splice variants provides a diverse family of transporters capable of translocating a wider range of antigen peptides from the cytosol to the ER and increasing the repertoire of MHC class I antigen complexes presented to the immune system. It is through such diversification mechanisms that it is now demonstrated that the antigen processing and presentation mechanisms of the immune system are able to drive and select T cell response diversity on the recognition side of the immune system, which is based on the enormous diversity of the T cell receptor (TCR).

Specific embodiments of the invention include novel splice variants of TAP1 and TAP2 and nucleic acids that encode them. Specific splice variants described herein are designated TAP1iso, TAP1iso^2, TAP1iso^3, TAP2iso and TAP2iso^2. The invention further encompasses novel TAP heterodimers ("TAPiso" heterodimers) incorporating the splice variants, exon polypeptides corresponding to previously unknown segments of the splice variants (i.e., corresponding to the exons or translated regions of genomic DNA by which the splice variants differ from known TAP1 or TAP2), vectors for cloning and expression of the splice variants or novel exon polypeptides, and recombinant host cells capable of expressing the splice variants, exon polypeptides or TAPiso heterodimers. The nucleotide sequence of *TAP2iso* and the deduced amino acid residue sequence of TAP2iso are shown in the Sequence Listing (SEQ ID NO: 4 and SEQ ID NO: 2, respectively).

The discoveries of the present invention also raise the possibility that a deletion or defect in expression of a particular TAP1 or 2 isoform, or that an abnormal expression level of one TAP1 or 2 isoform with respect to another, may cause the manifestation of autoimmune disease. For example, the ratios of TAP2 isoforms co-expressed by individuals was compared: Five patients diagnosed with Type I diabetes and five uneffected non-diabetic individuals were tested for levels of expression of TAP2 and TAP2iso. It was observed that, whereas in the non-diabetic individuals

expression levels were about the same, in 4 out of 5 of the diabetic patients, expression of TAP2iso predominated. This supports the use of the discoveries disclosed herein for diagnosis of TAP-associated conditions, in this case Type I diabetes, be measuring co-expression levels of the various TAP isoforms, for example the ratio of TAP2iso:TAP2. Methods for diagnosis and methods of treatment of such diseases associated with defective TAP isoform expression are in more detail below.

An isolated nucleic acid (polynucleotide) that encodes the mature TAP2iso polypeptide having the deduced amino acid sequence of SEQ ID NO: 2 was deposited under the Budapest Treaty with ATCC (American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (US)) under accession no. 209640 on February 24, 1998.

The ATCC microorganism deposit referred to above will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for purposes of Patent Procedure. The deposit is provided as a convenience to those of skill in the art and is not to be construed as an indication or admission that such a deposit is required under 35 U.S.C. §112 or is necessary for a complete understanding of the invention. The sequence of the polynucleotides contained in the deposited material, as well as the amino acid sequence of the polypeptides encoded thereby, is incorporated herein by reference and is controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited material, and no such license is hereby granted.

Isolation of TAP Subunit Splice Variants

Nucleic acids coding on expression for TAP1 or TAP2 splice variants according to this invention may be isolated by screening a cDNA library, such as a human splenocyte cDNA library. cDNA libraries may be screened using an oligonucleotide probe complementary to an exon of *TAP1* or *TAP2* (or, also, a probe complementary to an intron portion suspected of being an exon in a splice variant). Construction of suitable cDNA libraries is well known in the art. Any portion of a *TAP1* or *TAP2* mRNA or cDNA may be used, however it is preferred that the probe be designed so as not to span exons and to include coding sequences that are thought to be used in all isoforms. Thus far, variation in the *TAP1* and *TAP2* coding sequences has been discovered at the 3' end of the sequences, and therefore it is preferred to screen using probes based on the first nine or ten exons of *TAP1* and *TAP2*. Probes based on all or part of exons 8, 9 or 10 of *TAP1* or *TAP2* are most preferred.

Additional TAP subunit splice variants may also be isolated using reverse transcription polymerase chain reaction (RT-PCR) techniques: Now that the existence of TAP splice variants has

been discovered and disclosed herein, additional coding sequences for splice variants can be discovered by analysis of PCR products from segments of total RNA and mRNA (see the examples, *infra*). Forward and reverse PCR primer pairs can be designed from genomic sequence information disclosed herein or obtained elsewhere. The RT-PCR experiments set forth herein were greatly assisted by genomic sequence information on human chromosome 6 (which includes the *TAP* genes) that was generously provided by Dr. John Trowsdale and colleagues at the Imperial Cancer Research Fund, London. Primer pairs can be selected to amplify any segment of the *TAP* loci or, in RT-PCR, any segment of cDNA derived from total RNA, however it is preferred to select primer pairs that are about 300-500 base pairs apart, so that disparate co-amplified products can be readily distinguished, e.g., by separation on an electrophoresis gel. It is preferred to employ overlapping primer sets for investigation of adjacent segments, and it is preferred to verify unexpected RT-PCR products by amplifying the same region using different primer sets.

The TAP splice variants described herein were first detected when attempts to isolate monoclonal antibodies recognizing either N-terminal or C-terminal epitopes of the known TAP subunits failed to yield C-terminal antibodies that would clear a lysate containing TAP subunits of all TAP products, whereas antibodies recognizing the N-terminus were capable of clearing solutions of all TAP products.

It was decided to examine *TAP1* and *TAP2* RNA samples using reverse transcription polymerase chain reaction (RT-PCR) analysis to see if plural messages were being generated. A series of forward and reverse primers were prepared for examining segments of the *TAP1* and *TAP2* coding sequences. Primers were selected to bracket relatively small segments of the genomic sequence (e.g., 300-500 bp apart), and overlapping primer sets were used (i.e., sets of primers suitable for PCR-amplifying overlapping segments of DNA).

TAP2iso cDNA was isolated from a human splenic cDNA library using an oligonucleotide probe (19 bp, SEQ ID NO: 3) complementary to exon 10 of the known *TAP2* coding sequence. The *TAP2iso* coding sequence is set forth in the Sequence Listing as SEQ ID NO: 4; the deduced amino acid sequence for TAP2iso is shown at SEQ ID NO: 2. The *TAP2iso* polynucleotide (SEQ ID NO: 4) encodes a polypeptide of 653 amino acids. Amino acids 1-644 are identical to a previously characterized TAP2 (SWISS-PROT: Q03519; SEQ ID NO: 15), and the rest of the C-terminal nine amino acids are encoded by an alternatively spliced exon 12 (SEQ ID NO: 5). The novel peptide corresponding to the coding sequence of exon 12 (SEQ ID NO: 5) is referred to as the exon 12 peptide (SEQ ID NO: 1).

TAP1iso[3] cDNA was isolated in a similar manner from a human B cell cDNA library using an oligonucleotide probe complementary to exon 8 of the known *TAP1* coding sequence. The

TAP1iso^3 coding sequence is set forth in the Sequence Listing as SEQ ID NO: 24 (nucleotides 47-1804 of the isolated clone); the deduced amino acid sequence for TAP1iso^3 is shown at SEQ ID NO: 25. The *TAP1iso*^{3} polynucleotide (SEQ ID NO: 24, nucleotides 47-1804) encodes a polypeptide of 586 amino acids. Amino acids 1-581 are identical to a previously characterized TAP1 (SEQ ID NO: 19), and the rest of the C-terminal five amino acids are encoded by DNA 3' to the exon 8 of TAP1 (see Fig. 11).

The polynucleotides of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequences for splice variant TAP isoforms according to the present invention will be partially identical and partially different (e.g., < 50 % homologous) to the known TAP coding sequences (i.e., *TAP1* or *TAP2*, SEQ ID NOS: 18 and 17, respectively). The *TAP2iso* coding sequence may be identical to the coding sequences shown in the Sequence Listing section (e.g., SEQ ID NO: 4) or identical to that of the deposited clone or may be a different coding sequence, which different coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptide. Likewise, the *TAP1iso*, *TAP1iso*^{2}, *TAP1iso*^{3} coding sequences may be identical to the coding sequences shown in the Sequence Listing section (e.g., SEQ ID NO: 44) or identical to those indicated in Fig. 9A or may be a different coding sequence, which different coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptide.

The present invention further relates to TAPiso transporter proteins, TAP1iso and TAP2iso splice variant proteins, and exon polypeptides. Such TAP1 and TAP2 splice variants will be partially identical and partially different (e.g., < 50% homologous) to the known TAP1 and 2 subunits (i.e., SEQ ID NOS: 19 and 15, respectively); and TAPiso transporter proteins will be correspondingly partly identical and partly different from the known TAP transporter protein that consists of the heterodimer formed from TAP1 and TAP2. Exon polypeptides corresponding to previously unknown exons will have coding and amino acid sequences differing substantially from any known exon of TAP1 or TAP2. Specifically contemplated are TAP2iso proteins or exon polypeptides that have the deduced amino acid sequences of SEQ ID NOS: 1 and 2 or that have the amino acid sequence encoded by the deposited cDNA, as well as fragments thereof encoding polypeptides having the same biological function or activity as such polypeptides. The TAPiso polypeptides of the present invention may be recombinant polypeptides (i.e., non-naturally occurring polypeptides produced using recombinant DNA techniques), natural polypeptides or synthetic polypeptides, preferably recombinant polypeptides.

Homologues of the TAP1iso, TAP1iso^2, TAP1iso^3, TAP1iso and TAP2iso^2 polypeptides described herein, as well as homologues to any subsequently discovered TAP isoforms, and homologues of exon polypeptides (i.e., peptides corresponding to the divergent sequences of such TAP isoforms), may be formed by substitution, addition or deletion of one or more amino acids employing methods well known in the art and for particular purposes known in the art, such as addition of a polyhistidine "tail" in order to assist in purification or substitution of one up to several amino acids in order to obliterate an enzyme cleavage site. Preferably such homologues will retain functionality as subunits able to dimerize with counterpart TAP or TAPiso subunits in order to provide a functional TAP (or TAPiso) protein capable of translocating peptides in a cell.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The present invention also provides vectors that include *TAPiso* polynucleotides of the present invention, host cells that are genetically engineered with vectors of the invention, polypeptides produced by culturing such genetically engineered host cells. Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the *TAPiso* genes. The culture conditions, such as temperature, pH and the like, are those suitable for use with the host cell selected for expression and will be apparent to the skilled practitioner in this field.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant DNA techniques or, preferably, for transfecting cells to augment their capability to translocate cytosolic peptides to the ER for complexing with MHC class I molecules and eventual antigen display. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host. The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the skill of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned LTR or SV40 promoter, the *E. coli.* lac or trp, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. In addition, expression vectors preferably will contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells, such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance for bacterial cell cultures such as *E. coli.*

The vector containing the appropriate *TAP* DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate host cells, there may be mentioned bacterial cells, such as *E. coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, such as yeast; insect cells such as *Drosophila* and Sf9; animal cells such as CHO, COS or Bowes melanoma; plant cells, etc. However, where the object of transfection of the host cell is to form operative intracellular TAPiso proteins to augment or restore the capability of a cell to transport a particular antigen peptide or group of peptides, then mammalian cells, such as CHO or COS cells, or more particularly human cell lines, such as T cell or B cell lines, T1 or T2 cells (see *infra*), HeLa cells, U-937 cells, EBV-immortalized human B cell lines, PACA cells, and the like, are much preferred. The selection of an appropriate host is deemed to be within the skill of those skilled in the art from the teachings herein. Many suitable vectors and promoters useful in expression of proteins according to this invention are known to those of skill in the art, and many are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). Any other plasmid or vector may be used as long as it is replicable and viable in the selected host cell.

Introduction of the vectors into the host cell can be effected by any known method, including calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (see Davis et al., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the TAP gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can

be synthetically produced by conventional peptide synthesis methods: For example, direct synthesis of the peptides of the invention may be accomplished using techniques including, preferably, solid-phase peptide synthesis, although solution-phase synthesis may also be used. In solid-phase synthesis, for example, the synthesis is commenced from the carboxy-terminal end of the peptide using an α-amino protected amino acid. t-Butyloxycarbonyl (Boc) protective groups can be used for all amino groups, though other protective groups are suitable. See, Stewart et al., Solid-Phase Peptide Synthesis (1989), W. H. Freeman Co., San Francisco; and Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963). Polypeptides according to the invention may also be prepared commercially by companies providing peptide synthesis as a service (e.g., BACHEM Bioscience, Inc., King of Prussia, PA; Quality Controlled Biochemicals, Inc., Hopkinton, MA). For full-length TAPiso proteins, recombinant production is most preferred, due to the practical limits and expense of protein synthesis; for smaller peptides such as exon peptides according to this invention, solid-phase synthesis or commercial synthesis will be most advantageous.

The TAP polypeptides and exon polypeptides of the present invention can be recovered and purified from recombinant cell cultures or other solutions by suitable methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography, and the like. High performance liquid chromatography (HPLC) can be employed for final purification steps. The novel polypeptides of the present invention may be a naturally purified products, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated.

Functional Activity of TAP Subunit Splice Variants

TAPiso proteins according to the present invention preferably exhibit the same general functionality as known TAP transporter proteins, namely, translocation within a cell of peptides from the cytosol into the ER. However, the discoveries detailed herein demonstrate that the specific functionality of different TAP proteins, formed from different pairings of TAP1 and TAP2 isotypes, can lead to different classes of peptides being translocated, or the same peptide being translocated preferentially or at a different rate in comparison to other peptides.

Several ways of measuring TAP protein functionality are discussed below:

A. Transport Efficiency

The rate at which different TAP isoforms transport cytosolic peptides into the ER (transport efficiency) is one way to distinguish between homologous isoforms and one basis for TAP-mediated diversity in the presentation of MHC class I antigen complexes. The transport efficiency of TAPiso proteins may be compared with that of previously characterized TAP proteins using experimental techniques such as those of the following examples.

The rate at which MHC class I antigen complexes exit the ER can be used as one measure of TAP protein transport efficiency. MHC class I does not leave the ER until and unless it complexes with an appropriate peptide. TAP transport of peptides is the sole source of cytosolic peptides in the ER. The level of a particular MHC class I antigen complex within the Golgi apparatus of a cell, therefore, is directly proportional to TAP transport of the antigen peptide into the ER.

A preferred method of measuring the rate at which MHC class I antigen complexes exit the ER is through the quantification of MHC class I proteins of the Golgi apparatus sensitive to endoglycosidase H (endo H). Glycosylation of MHC class I proteins in the Golgi apparatus results in an increase in their molecular mass and an increase in resistance of MHC class I-linked glycans to endo H. By [^{35}S]methionine pulse-labeling of TAP-expressing or TAPiso-expressing cell cultures and chasing with unlabeled methionine at various times, the rate of MHC class I maturation in the ER can be gauged by observing differences in endo H sensitivity, e.g., compared with a TAP-deficient cell line such as T2. (See, Fig. 4A and 4B.)

B. Peptide Selectivity

Differential peptide binding specificity (peptide selectivity) is another way in which TAP isoforms may differ. All previously studied human allelic TAP polymorphs have shown no differences in peptide selectivity, however the nonallelic splice variants of the present invention have shown differences in peptide selectivity in comparison with previously studied TAP proteins.

The peptide binding domain of TAP proteins requires both a TAP1 and a TAP2 subunit. TAPiso transporter proteins including at least one TAPiso splice variant as disclosed herein, may result in the binding and translocation of a distinct class of cytosolic peptides, compared to those bound and translocated by a TAP1/TAP2 complex. This differential peptide selectivity has been demonstrated for TAP1/TAP2iso in comparison to TAP1/TAP2 (see examples, *infra*), thus the co-expression of TAP1 and TAP2 isoforms leads to diversification in peptides loaded into MHC class I molecules for antigen presentation at the cell surface.

The function of TAP proteins and the transport of different peptide repertoires can be tested by supplying known peptides, which may be labeled for ease of detection, to TAP and TAPiso

transformants and comparing their abilities to transport peptide into the ER and the rate at which transport is effected. Test peptides will preferably be of an optimal length for transport, i.e., about 6-25 amino acids in length, most preferably 8-12 amino acids in length. In a preferred assay of translocation, the test peptides are labeled with radioactive iodine (^{125}I). Additionally, glycosylation of the peptides within the ER of the MHC class I antigen complex can be monitored as an indication that successful transport and maturation of a properly formed antigen complex is taking place. One preferred method of monitoring maturation is noting increased resistance to endoglycosidase H (endo H), the principle being that as N-linked glycosylation of the MHC class I molecule proceeds to completion, the glycan structures of the protein become less susceptible to endo H cleavage.

Competitive inhibition techniques can also be used to compare translocation specificities of different TAP proteins. In such assays, two or more test peptide moieties are provided for possible transport by TAP proteins. Preferential TAP transport of a test peptide is measured by the competitive inhibition of the peptides in relation to each other. For example, one test peptide is detectably labeled (e.g., ^{125}I-labeled), and a second test peptide, labeled or unlabeled, is introduced into the cell, and the effect of the second peptide on the detectable translocation of the first test peptide is measured.

C. Restoration of Surface MHC Class I Antigen Complex Presentation

Functional TAPiso proteins of the present invention will bind and translocate peptides into the ER for MHC class I loading and eventual cell surface presentation. One preferred technique to measure the functionality of TAP1 and TAP2 isoforms, therefore, is to measure restoration of MHC class I antigen presentation in a TAP-deficient cell line, such as T2 cells (Attaya et al., Nature, 355:647 (1992)). A TAP-deficient cell can be transfected with *TAP1* and *TAP2* genes and/or genes encoding TAP splice variants to express a functional TAP or TAPiso transporter protein, and the extent of restoration of surface MHC class I antigen presentation on the transfected cell can then be measured.

Uses for TAP Subunit Splice Variants

The *TAPiso* genes described herein, and the TAPiso expression products and related exon polypeptides, will have many uses in the field of immunology and immunotherapy. For example, the *TAPiso* genes may be used to prepare recombinant host cells expressing only a particular TAPiso transporter protein, which cell will be useful in defining the specificity and translocation efficiency of particular TAPiso proteins. The TAPiso genes also may be used to produce useful quantities of isolated TAP1iso or TAP2iso proteins, which may be used as immunogens for the production of

monoclonal or polyclonal anti-TAP subunit antibodies or anti-exon polypeptide antibodies for use as diagnostic reagents or cell classification markers.

The *TAPiso* expression products or their fragments (including exon polypeptides), or cells expressing them, can be used as immunogens to produce antibodies recognizing such products or fragments. These antibodies can be, for example, polyclonal, monoclonal, chimeric, single-chain, Fab fragments, or the product of a Fab expression library. Various procedures are known in the art for the production of such antibodies. Antibodies generated against the polypeptide corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptide into an animal or by administering the polypeptide to an animal, preferably a nonhuman animal. The antibody so obtained will then recognized or bind to the polypeptide itself. In this manner, even a sequence encoding only a fragment of a TAP1iso or TAP2iso polypeptide can be used to generate antibodies recognizing the intact native polypeptide. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide or to detect expression of a particular TAP subunit or formation of a particular TAP or TAPiso transporter protein in particular cells or tissues. Moreover, a panel of anti-TAP1 or anti-TAP2 or anti-TAP heterodimer antibodies, specific to a range of TAP subunits and/or TAP heterodimers, can be used to identify and differentiate tissues and disease states corresponding to differential expression of TAP subunits or differential formation of TAP transporter proteins.

For preparation of monoclonal antibodies, any technique that provides antibodies produced by continuous cell line cultures can be used. Well known examples of such techniques include the hybridoma technique (Kohler and Milstein, Nature, 256:495-497 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today, 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 (1985)).

Techniques described for the production of single-chain antibodies in U.S. Patent No. 4,946,778 can be adapted to produce single-chain antibodies recognizing immunogenic polypeptide products of this invention.

Such antibodies can be used in methods relating to the localization and activity of the *TAP* expression products described herein, e.g., for imaging these proteins, measuring levels thereof in appropriate physiological samples, and the like.

The TAPiso polynucleotides of the present invention also provide a means for screening candidate drugs to identify those capable of enhancing or inhibiting peptide transport. Such candidate drugs would be useful in upregulating or downregulating the immune response to particular antigens or for treating disorders associated with a particular ratio of TAP expression

products produced by an individual. For example, where an immune disorder is associated with higher than normal expression of a particular TAP protein or a higher than normal expression in relation to another TAP protein, then modulation of one or the other expression products to correct the expression or balance the relative expression of TAP proteins can alleviate the manifestation of the disorder. For drug candidate screening, a mammalian cell or membrane preparation expressing one or more TAP proteins can be incubated with labeled peptides in the presence of the drug candidate. The ability of drug to change the rate of translocation of the peptides by the TAP protein(s) or to block the translocation can then be measured.

The *TAPiso* polynucleotides of the present invention may also be employed in accordance with the present invention for causing expression of TAPiso polypeptides *in vivo*, which is often referred to as "gene therapy". For example, cells from a patient may be transfected with a polynucleotide (DNA or RNA) encoding a TAP polypeptide *ex vivo*, using known transfection techniques. The resultant transfectants expressing the introduced polynucleotide can reverse a TAP deficiency in the host cell, or supplement low expression of a particular subunit by the host cell, or provide an additional TAP transporter protein in the cell, thus augmenting the repertoire of MHC class I antigen complexes that are displayed by the host cell. The transfectants then can be provided to a patient to be treated. Such gene therapy methods are well known in the art.

Similarly, cells may be engineered *in vivo* for expression of a TAP polypeptide *in vivo* by procedures known in the art. For instance, a cell producing a retroviral particle containing RNA encoding the TAP polypeptide of interest may be administered to a patient for infection and transformation of the patient's cells *in vivo* and expression of the particular TAP polypeptide *in vivo*. Alternatively, known microinection techniques can be employed to insert plasmid DNA into cells of a patient, thereby augmenting the class I antigen display of those cells and prompting a desired immune response. These and other methods for administering the polynucleotides or using a polypeptide of the present invention will be apparent to those skilled in the art from the foregoing description and the examples to follow.

In addition to gene therapy, the TAP splice variants disclosed herein may be used in improving vaccine designs or in overcoming weak immune responses in an individual to a pathogen. For example, there may be viruses that are able to avoid immune detection and attack in certain individuals because of inadequate transport of viral antigen peptides into the ER for complexing with MHC class I molecules and ultimate presentation on the cell surface. Such viruses expose a "hole" in the immune system, i.e., a gap in the antigen presentation repertoire that leads to an inability of the immune system to recognize and respond to the viral antigens, even though there are T cells bearing receptors expressed by the individual capable of binding the antigens if properly

presented. Where such inadequate transport of viral antigen peptides is the result of low expression or no expression of particular TAP variants, the *TAP1* and *TAP2* genes disclosed herein can be used to broaden the immune responsiveness of an individual. A preferred method for broadening an individual's immune responsiveness is by *ex vivo* transfection of MHC class I expressing cells, preferably lymphocytes, most preferably B cells, macrophages or dendritic cells, with DNA encoding TAP isoforms that are not expressed or are expressed at low levels, followed by reintroduction of the transfected cells into the individual, where the expression of additional TAP isoforms leads to presentation of additional antigen peptides, which, in turn, drives selection and proliferation of responsive T cells. The DNA encoding the non-expressed or inadequately expressed TAP isoform used in transfection may be specific or non-specific for the antigens derived from a particular pathogen, that is, the range of antigen peptides and thus the repertoire of displayed antigen complexes will be broadened by transfecting DNA encoding any supplementary isoform of TAP, but alternatively, if it is determined what antigen peptides result from proteolysis of a particular virus and if it is determined that the function of a particular TAP isoform leads to the translocation of such antigen peptides, then the transfection using the appropriate TAP isoform-encoding DNA will tailor the broadened immune response to the particular virus by causing an increase in the MHC class I/viral antigen complexes being presented by transfected cells. The effect of this vaccination will also continue past the cell *in vivo* life of the transfectants, because transient presentation of the supplemental repertoire of viral antigens will establish a T cell memory, and the individual challenged subsequently by the same virus will be able to mount a classic secondary antigen response.

This approach can also be used to address infectious diseases, especially where an individual's susceptibility to a disease is due to inadequate expression of a particular TAP isoform. In this way an individual can also be vaccinated to fight malignancies that escape immune attack due to inadequate presentation of antigen peptides associated with the malignant tissues. This approach would be especially useful in cases where a family history revealed a predisposition for developing certain cancers, such as breast cancer. If individuals from that family also showed abnormal expression of any of the TAP isoforms, transfected cell therapy could be used to correct the TAP deficiency or to balance the levels of expression of TAP isoforms to reflect normal levels.

Isolation, testing and use of particular TAP splice variants of the present invention will be further illustrated in the following examples. The specific parameters included in the following examples are intended to illustrate the practice of the invention, and they are not presented to in any way limit the scope of the invention.

EXAMPLE 1: TAP2iso

Described below is the isolation and characterization of a novel TAP2 splice variant (i.e., TAP2iso) that, when complexed with TAP1, forms a novel TAP heterodimer (TAP1/TAP2iso) distinct from the known TAP transporter protein. TAP2iso lacks TAP2 exon 11 and contains a newly-identified TAP2 exon 12, located 6533 base pairs (bp) downstream of TAP2 exon 11 (see Fig. 1). The 2496 bp full-length cDNA of TAP2iso (see SEQ ID NO: 4) predicts a protein of 653 amino acids (SEQ ID NO: 2), the last nine of which are encoded by exon 12 (Figs. 2 and 3). The protein encoded by this cDNA shows the ability to dimerize with TAP1 and to form a peptide-transporting heterodimer, however that TAP1/TAP2iso transporter exhibits characteristics that differ from those of the previously identified TAP.

Isolation of TAP2iso

The existence of heterofunctional TAP1 and 2 isoforms was detected during the course of work to isolate antibodies to the C-terminus of each of the known TAP subunits, TAP1 and TAP2. Whereas polyclonal antibodies recognizing epitopes of the N-terminal sequences of TAP1 and TAP2 had been isolated which would clear a cell lysate of all TAP expression products, polyclonal antibodies raised against the known C-terminal sequences were unable to remove all TAP proteins from a solution. One explanation of this phenomenon was that there were variant isoforms of the known TAP subunits differing at the C-terminal end to a sufficient degree that they could not be recognized by antibodies raised against only the known C-terminal sequences. Since a polyclonal antiserum would be expected to clear all of the known polymorphs of TAP1 and TAP2, it was decided to search for TAP1 and 2 splice variants exhibiting entirely heterologous C-terminal sequences compared to the known TAP1 and 2 proteins.

It was decided to examine a cDNA library with probes complementary to a sequence coding for an upstream segment of the TAP1 and TAP2 proteins, to see if variant transcripts could be detected. Probes were designed using sequence information of the entire locus including the known *TAP* genes, generously provided by Dr. John Trowsdale and colleagues at the Imperial Cancer Research Fund, London (GB). When a specific 19-base pair (bp) oligonucleotide probe complementary to exon 10 of *TAP2* was used to screen a human spleen cDNA library prepared from a single individual, two different full-length transcripts were detected and analyzed.

The human splenic cDNA library was screened with the use of a soluble hybridization system (Gene Trapper; Life Technologies, Inc., Gaithersberg, Maryland (US)). The specific oligonucleotide probe (5'-ATGTAGGGGAGAAGGGAAG-3', identified as SEQ ID NO: 3) targeted to exon 10 of *TAP2* was synthesized and purified by electrophoresis on a 12% polyacrylamide gel

[acrylamide: bisacrylamide, 19:1 (w/w)] containing 8 M urea with 1X Tris-borate-EDTA buffer. The probe (3 μg) was biotinylated with the use of terminal deoxynucleotidyl transferase and biotin-conjugated deoxycytidine triphosphate. The cDNA library was digested for 25 minutes at 25° C with Gene II (Gibro-BRL), an enzyme that introduces random nicks into DNA, and then for 60 minutes at 37° C with *Escherichia coli* exonuclease III to generate single-stranded plasmid DNA. Hybridization between single-stranded plasmid DNA and 20 ng of the biotinylated probe was performed in solution for 60 minutes at 37° C. The mixture was then incubated for an additional 20 minutes with streptavidin-coated magnetic beads, after which the beads were separated and the hybridization complexes eluted.

The single-stranded cDNA was converted to double-stranded DNA by incubation for 15 minutes at 70° C in a final volume of 30 μl containing 2U of Taq polymerase, 20 ng of non-biotinylated probe as primer, and 200 nM of each deoxynucleoside triphosphate. The double-stranded DNA was inserted into a cloning vector (PREP 8, Invitrogen) having an ampicillin resistance marker, and *Escherichia coli* were transformed with 3 μl of the resulting DNA by electroporation at 1800 V, 25 μF, and 100 ohms. The bacteria were plated onto four agar plates containing ampicillin (100 μg/ml). Positive colonies were sequenced by primer walking.

The sequence of one full-length *TAP2* clone, 0123, was identical in the 5' untranslated region and in exons 1 through 10 to that of previously characterized *TAP2* cDNAs as well as to that of other *TAP2* clones (such as clone 0121) isolated from the same library. However, clone 0123 lacked exon 11 and the 3' untranslated region of the other known *TAP2* cDNAs and contained a new 27 bp exon (exon 12) and 3' untranslated region. The exon 12 sequence is present in the *TAP2* genomic sequence 6533 bp downstream of exon 11. Sequencing of the predicted splice sites confirmed the presence of functional motifs to allow the splicing of exon 10 to exon 11 (clone 0121) and exon 10 to exon 12 (clone 0123). Of the 26 informative cDNAs isolated using the exon 10 probe (SEQ ID NO: 3), 9 corresponded to the new splice form, which was designated *TAP2iso*.

The predicted TAP2iso protein encoded by *TAP2iso* contains 653 amino acids, compared with 703 amino acids for the previously characterized TAP2; the COOH-terminal nine residues of *TAP2iso* are encoded by exon 12. Alignment of amino acid sequence, hydrophobicity and secondary structure were analyzed by GCG program-SEG, MAP, TRANSLATION and PEPPLOT (Genetics Computer Group, Madison, WI). Hydrophobicity analysis revealed a higher β factor for TAP2iso and in contrast a greater α factor for TAP2.

Vector Contruction and Transfection

The *TAP1* (*TAP1 A* allele) cDNA was removed from the pCMV-SPORT vector (Gibco-BRL) by *BamH*I and *Sal*I digestion and inserted into the PREP 4 vector (Invitrogen) at the *BamH*I and *Xho*I sites. *TAP2* (clone 0121, *TAP2 F* allele) and *TAP2iso* were removed from pCMV-SPORT vector by digestion with *Not*I and *Kpn*I and inserted into the PREP 8 vector (Invitrogen) at the *Not*I and *Kpn*I sites.

Plasmid DNAs were prepared using MAXI-PREP kits (Qiagen). *TAP1*- and *TAP2*-deficient T2 cells (donated by P. Cresswell, Howard Hughes Medical Institute, Yale University) (1-2 $\times$ 10^7 cells/ml) were transfected by electroporation with 10 μg of *TAP1* and *TAP2* or *TAP2iso* linear plasmid DNA in a 0.4-cm cuvette at 260 V and 960 μF. Subcloning and selection of transfectants were performed by culturing in the presence of hygromycin (250 μg/ml) or histidinol (2 mM) for the PREP 4 and PREP 8 vectors, respectively.

Stable transfectant clones were analyzed by indirect immunofluorescence as described in Fu et al., J. Clin. Invest., 91:2301 (1993), with an anti-HLA class I (A2) murine monoclonal antibody (clone 0791HA; One Lambda, San Diego, California (US)) and an anti-HLA class II murine monoclonal antibody (clone L243, ATCC accession no. HB55, Manassas, Virginia (US)). Immune complexes were detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibodies (Coulter, Hialeah, Florida (US)) and an Epics Elite flow cytometer (Coulter).

Transport Efficiency of TAP2iso in the Transport of Peptides to the ER

The rate at which MHC class I antigen complexes exit the ER was measured in T2 cells stably transfected with *TAP1* and either *TAP2* or *TAP2iso*, as a measure of TAP1/TAP2iso transport efficiency in comparison to TAP1/TAP2. T2 cells were transfected with *TAP1* and *TAP2iso* cDNAs, harvested at 4° C and cultured for 30 minutes in methionine-free medium. High density cells (1 $\times$ 10^8 cells/ml) were labeled for 15 minutes in the presence of 500 μCi [^{35}S]methionine (Amersham), and then chased at the indicated times in the presence of 10 mM unlabeled methionine.

Cells were lysed in an ice-cold lysis buffer, and lysates were incubated overnight at 4° C with protein A Sepharose beads (Pharmacia) and normal rabbit serum (1:200 dilution). The beads were removed by centrifugation and the resulting supernatant was incubated for 12 hours at 4° C with protein A beads and monoclonal antibody W6/32 (American Type Culture Collection (ATCC) accession no. HB 95, Manassas, Virginia (US)), which recognizes HLA class I molecules. The beads were separated by centrifugation and washed extensively, after which proteins were eluted from the beads by boiling for 5 minutes in SDS sample buffer and analyzed by 12.5% of SDS

polyacrylamide gel electrophoresis. The gel was treated with enhancer, dried, and exposed to X-ray film for 12 to 72 hours.

The results are shown in Figures 4A and 4B. Samples were incubated in the absence (-) or presence (+) of endo H as described in Neefjes et al., Eur. J. Immunol., 25:1133 (1995). "r" and "s" indicate HLR class I proteins resistant and sensitive to endo H, respectively. The results revealed that TAP2iso (Fig. 4A) and TAP2 (data not shown) both expressed with TAP1 showed increasing HLA class I maturation as reflected in decreasing endo H sensitivity to similar extents relative to that apparent in untransfected T2 cells (Fig. 4B).

Peptide Selectivity of TAP2iso in the Transport of Peptides to the ER

The peptide selectivities of TAP1/TAP2 and TAP1/TAP2iso heterodimers were compared by measuring the transport of ^{125}I-labeled peptides into the ER of transfected T2 cells permeabilized with streptolysin O. Peptides that have entered the ER were detected on the basis of their consequent glycosylation.

The transport of three different peptides was measured: Test Peptide RRYQNSTEL (SEQ ID NO: 6), which is a variant of a peptide eluted from HLA class I B27 (Androlewicz et al., Proc. Natl. Acad. Sci. U.S.A., 91:12716 (1994)), with a polar asparagine substituted for a charged lysine at position 5 to produce an NXT motif for glycosylation; Test Peptide IYLGPFSPNVTL (SEQ ID NO: 7); and Test Peptide TVDNKTRYE (SEQ ID NO: 8), which is transported efficiently by the product of the rat *TAP2*^{a} allele but poorly by that of the rat *TAP2*^{u} allele (Momburg et al., Nature, 367:648 (1994); Fu et al., J. Clin. Invest., 91:2301 (1993); Ortmann et al., Nature, 368:864 (1994)). The size of all test peptides fell within the range of 8-12 amino acids that is optimal for transport by all TAP proteins characterized to date.

The test peptides were synthesized by Quality Controlled Biochemical, Inc. (Hopkington, MA), and their sequences were confirmed by mass spectrometry. The purity of peptide preparations was >95% as judged by high-performance liquid chromatography. 10 mM stock solutions of peptides were prepared in dimethyl sulfoxide. Peptides (25 µg) were directly iodinated at unique tyrosine residues with the use of chloramine T (Sigma, St. Louis, MO), and free iodine was removed by gel filtration using Sephadex G10 (Pharmacia) column chromatography. The specific activity of the ^{125}I-iodinated peptides ranged from 20 to 50 cpm/fmol.

Glycosylated ^{125}I-labeled Peptide RRYQNSTEL (SEQ ID NO: 6), Peptide IYLGPFSPNVTL (SEQ ID NO: 7), or Peptide TVDNKTRYE (SEQ ID NO: 8) was recovered and measured . The peptide translocation assay was performed essentially as described by Neefjes et al., in Science, 261:769 (1993) and Momburg et al., in J. Exp. Med., 179:1613 (1994). Briefly, 2.5-5.0 × 10^6 T2

cells (transfected or untransfected) were washed once with incubation buffer (130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EDTA, 2 mM $MgCl_2$, 5 mM Hepes, pH 7.3) and permeabilized for 10 minutes at 37° C with streptolysin O (2 IU/ml) (Wellcome, Beckenham, UK) in 50 µl of incubation buffer. After further addition of 10 µl of 100 mM adenosine triphosphate, 10 µl of ^{125}I-labeled peptide (~15 pM), and 30 µl of incubation buffer, cells were incubated for an additional 10 minutes at 37° C. Transport was terminated by the addition of 1 ml of 1% NP-40 detergent, after which nuclei were removed by centrifugation and glycosylated peptides were recovered using concanavalin A-Sepharose (Sigma, St. Louis, Missouri (US)) and quantitated with a gamma counter (LKB-Wallac).

The results are shown in Figure 5 as means ± SD of 3 independent experiments with 10 different clones. Peptide RRYQNSTEL was transported to similar extents by TAP1/TAP2 and TAP1/TAP2iso, while Peptide TVDNKTRYE was transported to a greater extent by TAP1/TAP2 than by TAP1/TAP2iso, and Peptide IYLGPFSPNVTL was tranported to a greater extent by TAP/TAP2iso than by TAP1/TAP2. The amount of translocated peptide is shown as the percentage of the input radioactive peptide. Untransfected T2 cells or those expressing TAP1 or TAP2iso alone did not show appreciable transport of any test peptide.

Differential peptide selectivity of the TAP1/TAP2 and TAP1/TAP2iso heterodimers was further investigated by competition experiments with ^{125}I-labeled Peptide RRYQNSTEL (SEQ ID NO: 6) and various unlabeled peptides: Peptide RRYQNSTEL (SEQ ID NO: 6); Peptide IYLGPFSPNVTL (SEQ ID NO: 7); Peptide TVDNKTRYE (SEQ ID NO: 8); Peptide SYSMEHGRWGKPVGKKRRPVKVYP (SEQ ID NO: 9), the S1-24 fragment of human adrenocorticotropic hormone); and Peptide RGFFYTPKA (SEQ ID NO: 10), residues 22 to 30 (human insulin B chain). The iodinated 9-mer Peptide RRYQNSTEL was translocated in the presence of different concentrations of unlabeled competition peptides. The glycosylated peptides were recovered by Con A-Sepharose and quantitated. Figures 6A-6E graphically illustate the results of: (6A) competition of ^{125}I-Peptide RRYQNSTEL with unlabeled Peptide RRYQNSTEL (self); (6B) competition of ^{125}I-Peptide RRYQNSTEL with Peptide IYLGPFSPNVTL; (6C) competition of ^{125}I-Peptide RRYQNSTEL with Peptide TVDNKTRYE; and (6D) competition of ^{125}I-Peptide RRYQNSTEL with Peptide SYSMEHGRWGKPVGKKRRPVKVYP; and (6E) competition of ^{125}I-Peptide RRYQNSTEL with Peptide RGFFYTPKA.

Unlabeled Peptide RRYQNSTEL inhibited transport of ^{125}I-labeled Peptide RRYQNSTEL in TAP1/TAP2 and TAP1-TAP2iso transfectants to similar extents; the median inhibitory concentration (IC_{50}) was ~0.15 µM in both instances (Fig. 6A). With unlabeled Peptide IYLGGPFSPNVL as competitor, transport of ^{125}I-Peptide RRYQNSTEL was inhibited to a markedly

greater extent in TAP1/TAP2iso transfectants than in TAP1/TAP2 transfected cells (Fig. 6B). With unlabeled Peptide TVDNKTRYE as competitor, transport of ^{125}I-Peptide RRYQNSTEL was inhibited to a markedly greater extent in TAP1/TAP2 transfectants than in TAP1/TAP2iso transfected cells (Fig. 6C). Peptide SYSMEHGRWGKPVGKKRRPVKVYP, a 24-residue peptide, did not inhibit transport of Peptide RRYQNSTEL in either transfectant to a substantial extent (Fig. 6D), which is consistent with previous studies of TAP transporters showing a preference for peptides of 8 to 12 amino acids. Finally, Peptide RGFFYTPKA inhibited the transport of Peptide RRYQNSTEL in TAP1/TAP2iso to a greater extent than that mediated by TAP1/TAP2 (IC_{50} values of 0.11 and 0.3 μM, respectively) (Fig. 6E). Thus, the two transporter heterodimers showed opposite preferences with regard to three of the five test peptides.

TAP2iso Restoration of MHC Class I Antigen Complex Surface Presentation in T2 Cells

The introduction of *TAP1* and *TAP2* genes into mutant T2 cells has been shown to restore the normal processing and surface expression of HLA class I molecules (Attaya et al., Nature, 355:647 (1992); Spies and DeMars, Nature, 351:323 (1991); and Powis et al., J. Exp. Med., 173:913 (1991)). Here, the functional properties of TAP2 and TAP2iso were compared by testing the effects of TAP2- or TAP2iso-containing TAP heterodimers on surface HLA class I antigen complex expression.

T2 cells were transfected with *TAP1* cDNA in combination with *TAP2* or *TAPiso* cDNAs as described above. The surface expression of MHC class I molecule A2 and the and MHC class II epitope recognized by monoclonal antibodies 0791HA and L243, respectively, were examined by flow cytometry in T1 cells or T2 cells transfected with TAP1 and either *TAP2* or *TAP2iso* cDNAs.

Flow cytometric analysis revealed that transfection of T2 cells with *TAP1* or *TAP2iso* cDNAs alone had no effect on surface expression of HLA class I (see Table 1, below). In contrast, transfection of T2 cells with *TAP1* and *TAP2* or *TAP1* and *TAP2iso* cDNA combinations resulted in restored surface expression of MHC class I. Surface MHC class I expression was ~30% greater in cells transfected with *TAP1* and *TAP2iso* cDNAs than in those transfected with *TAP1* and *TAP2* cDNAs. Consistent with the fact that T2 cells have a large homozygous deletion that encompasses both *TAP* and MHC II genes, only parental T1 cells were stained with antibodies to human MHC class II (Fig. 7).

Table 1. Restoration of surface expression of HLA class I (A2) on T2 cells transfected with both *TAP1* and either *TAP2* or *TAP2iso* cDNAs.

Cell Line	mRNA *TAP1*	mRNA *TAP2*	mRNA *TAP2iso*	Mean fluorescence of HLA class 1 (A2)* Mean ± SD
T1	(+)	(+)	(+)	8.43 ± .21
T2				5.26 ± .20
T2 (TAP1/2iso)	(+)		(+)	11.16 ± .50†
T2 (TAP1/2)	(+)	(+)		7.59 ± .31
T2 (TAP2)			(+)	6.11 ± 1.09
T2 (TAP1)	(+)			5.19 ± .35

*Data are means ± SD for four separate experiments performed over a 6-month period with at least three stable clones for each transfection group. †P<0.001 vs. T2 cells transfected with *TAP1* and *TAP2* cDNAs.

TAP2iso Expression in Various Human Cell Lines

Reverse transcription polymerase chain reaction (RT-PCR) analysis and sequencing of the RT-PCR products was conducted to determine the presence of TAP2iso expression in various human cell lines.

RT-PCR was performed with total RNA prepared from the various cell lines with Trizol reagent (Gibco-BRL). For the *TAP2* and *TAP2iso* cDNAs, PCR was performed with a shared primer targeted to exon 10 (SEQ ID NO: 3) and two different antisense primers targeted to the 3' untranslated region following exon 11 (SEQ ID NO: 11) and the 3' untranslated region following exon 12 (SEQ ID NO: 12), respectively. β_2-microblobulin RNA was amplified for a RT-PCR control, and the primers used are shown at SEQ ID NO: 13 and SEQ ID NO: 14.

First strand cDNA was synthesized from 3 μg of RNA by incubation at 42° C for 50 minutes in a final volume of 50 μl containing 200 U of Superscript II reverse transcriptase (Gibco-BRL), 0.05 μg of oligo(dT), and 200 nM of each deoxynucleoside triphosphate. A portion (1.5 to 3.0 μl) of the reaction mixture was then subjected to PCR in a final volume of 50 μl containing 0.1 nM of each primer, 200 nM of each deoxynucleoside triphosphate, and 2.5 U of Taq polymerase. After an initial denaturation step of 94° C for 2 minutes, amplification was performed for 36 cycles of 94° C for 30 sec., 57° C for 30 sec. and 72° C for 1 min. PCR products were analyzed by agarose gel electrophoresis and sequenced using DNA polymeras (Sequenase 2.0, Amersham Life Science, Arlington Heights, Illinois (US)).

Results are shown in Fig. 3. RT-PCR analysis revealed the presence of both *TAP2* and *TAP2iso* mRNAs in human fresh peripheral blood lymphocytes (lanes 3 and 4), Epstein-Barr virus-immortalized B cell lines (lanes 5 and 6), MOLT-4 acute lymphoblastic leukemia cells (lane 7), THP-1 monocytic cells (lane 8), U-937 histocytic lymphoma cells (lane 9), HeLa epithelioid carcinoma cells (lane 10), and PACA pancreatic carcinoma cells (lane 11). The presence of *TAP2* and *TAP2iso* mRNAs in T1 cells, a human lymphoblastoid B cell line (lane 1), but not in T1-derived T2 cells (lane 2), which contain a large homozygous deletion of the HLA class II region that encompasses the *TAP1* and *TAP2* genes, confirmed the specificity of the RT-PCR and was also consistent with *TAP2iso* mRNA being a splice product of *TAP2* rather than being derived from a distinct gene located elsewhere in the genome.

EXAMPLE 2: Detection of TAP1iso and TAP1iso^2

Multiple samples of cDNA were analyzed using PCR primers designed using genomic sequence information of the DNA linking the coding regions of the 3' exons known for TAP1 DNA, i.e., exons 9, 10 and 11 (and the introns). Two PCR primers were designed: The forward primer (from exon 9) was: 5'-TAGTTTCATCTCTGGACTCCCTCA-3' (SEQ ID NO: 20); the reverse primer (from the intron following exon 10) was: 5'-AGGTGTCTTTGCCTCGTCTTCT-3' (SEQ ID NO: 21). Total RNA, mRNA and cDNA samples were prepared from various cell lines. First strand cDNAs were synthesized from 3 μg of RNA or 0.5 μg of mRNA by incubating at 42° C for 50 min. in a final volume of 50 μl. A portion of the reaction mixture was then subjected to PCR with the above set of primers. PCR products were then analyzed by agarose gel eletrophoresis, and some samples were recovered for DNA sequencing. Analysis of the PCR products revealed the presence of two unexpected PCR products not corresponding to the expected *TAP1* product but present in all human cell samples tested. This indicated two additional splice variants corresponding to TAP1, and these were designated TAP1iso and TAP1iso^2. The fact that the two variants appeared in all samples indicated that the variants were not allelic polymorphs but were variants of the original gene.

Referring to Fig. 8, results of electrophoresis of the RT-PCR products from various samples using the above primers are shown. Lane 1 shows molecular weight markers; lane 2 shows PCR products from genomic DNA of and EBV-immortalized B cell line from a first individual (Control #1); lane 3 shows RT-PCR products from nuclear mRNA from the same Control #1 cell line; lane 4 shows RT-PCR products from cytoplasmic mRNA from Control #1; lane 5 shows RT-PCR products from total RNA from Control #1, without DNAase; lane 6 shows RT-PCR products from total RNA from Control #1, with DNAase digestion; lane 7 shows RT-PCR products from cytoplasmic mRNA

of an EBV-immortalized B cell line from a second individual (Control #2), with DNAase digestion; lane 8 shows RT-PCR products from cytoplasmic mRNA of an EBV-immortalized B cell line from a third individual (Control #3), with DNAase digestion; lane 9 shows RT-PCR products from cytoplasmic mRNA of an EBV-immortalized B cell line from a fourth individual (Control #4), with DNAase digestion; and lane 10 shows RT-PCR products from cytoplasmic mRNA of an EBV-immortalized B cell line from a patient with type I diabetes.

As can be seen in the figure, the high molecular weight PCR product amplifying the entire sequence spanning the intron between exons 9 and 10 is clearly visible in samples including genomic DNA or unspliced RNA (lanes 2, 3 and 5). Cytoplasmic and nuclear mRNA are compared (e.g., lanes 3 and 4) to confirm that the unexpected products are not amplified mRNA that has not been completely spliced. Comparison of cytoplasmic mRNA from several individuals (e.g., lanes 4 and 6-10) confirms that the TAP1iso and TAP1iso^2 products are splice variants and not random mutations. The relative intensities of the signals is intriguing, in that it suggests that expression ratios may differ to some extent from individual to individual, and the equal, intense bands shown in the diabetic subject (lane 10) suggest a possible link between TAP1 isoform expression and the disease (see, also, Example 4, *infra*).

Sequencing of the TAP1iso and TAP1iso^2 bands showed that TAP1iso represented a splice form containing exon 9, exon 10 and retaining intron 10 at least to the primer location, and that TAP1iso^2 contained both exons 9 and 10 but a smaller portion of retained intron 10. A comparison of the PCR products is shown in Fig. 9A. A schematic diagram of the relationship between genomic DNA and the DNA indicated by the PCR products is shown in Fig. 9B. The heavy-lined area of intron 10 indicates the section of the intron common to both splice variant PCR products, up to the primer boundary.

EXAMPLE 3: Detection of TAP2iso^2

Using methods similar to those described above, cDNA derived from cytoplasmic mRNA of PBLs from multiple human donors was amplified and analyzed by gel eletrophoresis to determine whether additional TAP2 splice variants could be detected using two exonic primers. The forward primer was: 5'-GCTACTAGTGCCCTAGATGTGCAGT-3' (SEQ ID NO: 22), located in TAP2 exon 10; and the reverse primer was: 5'-CTTCTGCAGCTTGCCCTCCTGGAG-3' (SEQ ID NO: 23), located in TAP2 exon 11.

Analysis of the PCR products showed that PBLs of all of the donors exhibited the fully spliced TAP2 message (lower arrow, Fig. 10) but that some donors also exhibited a larger product (middle arrow, Fig. 10) with presumed partial retention of intron 10 sequence. This second product

indicated a second TAP2 splice variant, which included at least portions of exons 10 and 11 and a portion of intron 10. This splice variant was designated TAP2iso^2. The control genomic DNA sample (lane 11, upper arrow) showed an expected very large molecular weight PCR product including amplified DNA that is present in neither of the TAP2 splice variants. Referring to Fig. 10, lane 1 shows the PCR products derived from cytoplasmic mRNA from a first individual; lane 2 shows the PCR products derived from cytoplasmic mRNA from a second individual; lane 3 shows the PCR products derived from cytoplasmic mRNA from a hypothyroid patient; lane 4 shows the PCR products derived from cytoplasmic mRNA from a third individual; lane 5 shows the PCR products derived from cytoplasmic mRNA from a fourth individual; lane 6 shows the PCR products derived from cytoplasmic mRNA from a fifth individual; lane 7 shows the PCR products derived from cytoplasmic mRNA from a sixth individual; lane 9 shows the PCR products derived from cytoplasmic mRNA from a seventh individual; and lane 10 shows the PCR product derived from a genomic DNA sample. The bands corresponding to the TAP2iso^2 product are not present in three of the normal individuals (lanes 2, 6 and 9); and the hypothyroid patient does not show any readily descernable abnormality associated with the expression of these two TAP2 isoforms.

EXAMPLE 4: TAP2 Expression Ratio Association With Diabetes

A further investigation of association between TAP1 or TAP2 isotype expression and disease states was conducted by testing five patients being treated for Type I diabetes and five unaffected, non-diabetic subjects. The relative expression in each of the subjects of TAP2 and TAP2iso was determined in a RNAse protection assay. All subjects were found to co-express TAP2 and TAP2iso. In four of five diabetic subjects, expression of TAP2iso greatly predominated over TAP2, where in all five non-diabetic subjects, the expression levels of TAP2 and TAP2iso were approximately even. These results and the results above indicate that some manifestations of autoimmune diseases such as diabetes may be shown to have an association with relative TAP isoform expression, and one or more aspects of TAP isoform expression may be observed to help diagnose or monitor the penetration of the disease.

Based on the discoveries herein and the links observed thus far between TAP diversity and some disease states, changes in the expression of one or moere TAP isoforms will also be indicative of certain infections and malignancies that avoid immune response by interruption of specific TAP expression. Thus, differential expression of one or more TAP isoforms may also be used to diagnose and monitor infectious diseases or cancers associated with reduction, upregulation or elimination of expression of a TAP protein.

EXAMPLE 5: Isoation of TAP1iso^3

Using the same techniques as described in Example 1, supra, a further TAP1 isoform cDNA (designated *TAPiso*^{3}) was isolated, using a probe targeted to TAP1 exon 8 and a cDNA library made from B lymphocytes obtained from a normal individual. The library was probed using the same solution hybridization technique.

The sequence of a full-length *TAP1* splice variant clone (SEQ ID NO: 24) was isolated which was identical in the 5' untranslated region and in exons 1 through 8 to that of previously characterized *TAP1* cDNAs. However, the new *TAP1* cDNA clone lacked exons 9, 10 and 11 and the 3' untranslated region of the other known *TAP1* cDNAs, and it contained coding sequence for an additional five amino acid residues in frame with the 3' end of exon 8, and a new 3' untranslated region. A schematic diagram of the genomic DNA including TAP1 exons 1-11, with diagrams of the TAP1 and TAP1iso^3 messages is illustrated in Fig. 11.

The predicted TAP1iso^3 protein (SEQ ID NO: 25) encoded by *TAP1iso*^{3} (coding sequence = nucleotides 47-1804 of SEQ ID NO: 24) contains 586 amino acids, compared with 748 amino acids for the previously characterized TAP1 (SEQ ID NO: 19).

By following the examples given above, it is contemplated that additional splice variants of the TAP1 and TAP2 proteins will be discovered and that their specificities for different types and repertoires of antigen peptides will be mapped out. The discovery of multiple splice variants having similar but not identical transport functionality expands previous views as to the limits of antigen display by MHC class I molecules. The mechanism by which cytotoxic T cells are alerted to somatic events requiring an immune response is now seen to be more complex than originally thought, and new methods for detecting predisposition to genetically linked autoimmune disorders can now be contemplated. For example, in a disease that is characterized by a deletion or a mutation preventing expression of one or more of the TAP splice variants, gene therapy to replace expression of the lost splice variant(s), or other therapy to restore surface expression of the peptide repertoire for which the lost splice variant is specific, will effectively treat the disease. Similarly, a virus that knocks out expression of a splice variant or alters the ratio of expression of two or more splice variants, may be diagnosed by detection of the change in splice variant expression and may be combatted using vaccines designed to overcome the reduction or elimination of the MHC class I antigen complexes that results from the virus-mediated changes in splice variant expression. Some tumors may also arise through alteration of splice variant expression, and thus certain cancers may be diagnosed by detecting altered splice variant expression and treated by correcting the expression or otherwise overcoming the change in MHC class I antigen complex display that results from the

altered expression. These and other obvious applications of the discoveries herein relating to TAP splice variants are contemplated and are intended to be included in the scope of the present invention.

Table of Sequence Identification Numbers (SEQ ID NOS:)

SEQ ID NO:	Description
1	Amino acid sequence of TAP2iso exon 12 polypeptide
2	Amino acid sequence of TAP2iso polypeptide
3	TAP2 exon 10 oligonucleotide probe
4	Nucleic acid sequence of *TAP2iso*
5	Nucleic acid sequence of *TAP2iso* exon 12
6	Test Peptide RRYQNSTEL
7	Test Peptide IYLGPFSPNVTL
8	Test Peptide TVDNKTRYE
9	Test Peptide SYSMEHGRWGKPVGKKRRPVKVYP
10	insulin β chain$_{22\text{-}30}$: RGFFYTPKA
11	TAP2 exon 11 oligonucleotide probe
12	TAP2 exon 12 oligonucleotide probe
13	β_2-microblobulin oligonucleotide probe
14	β_2-microblobulin oligonucleotide probe
15	Amino acid sequence of TAP2 polypeptide
16	Amino acid sequence of TAP2 exon 11 polypeptide
17	Nucleic acid sequence of *TAP2*
18	Nucleic acid sequence of *TAP1*
19	Amino acid sequence of TAP1 polypeptide
20	Nucleic acid sequence of TAP1 exon 9 primer
21	Nucleic acid sequence of TAP1 intron 10 primer
22	Nucleic acid sequence of TAP2 exon 10 primer
23	Nucleic acid sequence of TAP2 exon 11 primer
24	Nucleic acid sequence of isolated clone including *TAP1iso*^{3} coding sequence
25	Amino acid sequence of TAP1iso^3 polypeptide

All of the publications cited herein are hereby incorporated by reference in their entirety.

Claims

1. An isolated nucleic acid encoding a TAP1 or TAP2 splice variant.
2. An isolated nucleic acid comprising:

   a polynucleotide sequence that is at least 95% identical to the sequence of a polynucleotide selected from the group consisting of

   (a) a polynucleotide encoding a polypeptide having the amino acid sequence of SEQ ID NO: 2,

   (b) a polynucleotide encoding a polypeptide having the amino acid sequence of SEQ ID NO: 25, and

   (c) the complement of (a) or (b).
3. The isolated nucleic acid of Claim 2, wherein said polynucleotide (a) has the sequence of SEQ ID NO: 4.
4. The isolated nucleic acid of Claim 2, wherein said polynucleotide (a) has the sequence of SEQ ID NO: 24.
5. An isolated nucleic acid encoding a TAP2 exon 12 polypeptide.
6. The isolated nucleic acid of Claim 5, wherein said nucleic acid has the sequence of SEQ ID NO: 5.
7. An expression vector comprising the nucleic acid of Claim 1.
8. An expression vector comprising the nucleic acid of Claim 2.
9. An expression vector comprising the nucleic acid of Claim 3.
10. An expression vector comprising the nucleic acid of Claim 4.
11. An expression vector comprising the nucleic acid of Claim 5.
12. An expression vector comprising the nucleic acid of Claim 6.
13. A host cell transfected with the vector according to Claim 7.
14. A host cell transfected with the vector according to Claim 8.
15. A host cell transfected with the vector according to Claim 9.
16. A host cell transfected with the vector according to Claim 10.
17. A host cell transfected with the vector according to Claim 11.
18. A host cell transfected with the vector according to Claim 12.
19. A process for producing a polypeptide comprising culturing the host cell of Claim 13 under conditions suitable to produce the polypeptide encoded by said polynucleotide.
20. A process for producing a polypeptide comprising culturing the host cell of Claim 14 under conditions suitable to produce the polypeptide encoded by said polynucleotide.

21. A process for producing a polypeptide comprising culturing the host cell of Claim 15 under conditions suitable to produce the polypeptide encoded by said polynucleotide.
22. A process for producing a polypeptide comprising culturing the host cell of Claim 16 under conditions suitable to produce the polypeptide encoded by said polynucleotide.
23. A process for producing a polypeptide comprising culturing the host cell of Claim 17 under conditions suitable to produce the polypeptide encoded by said polynucleotide.
24. A process for producing a polypeptide comprising culturing the host cell of Claim 18 under conditions suitable to produce the polypeptide encoded by said polynucleotide.
25. A method of altering transport of peptides from the cytosol to the endoplasmic reticulum of a living cell comprising: transfecting a living cell with a vector according to Claim 7, then culturing the transfected cell under conditions suitable to produce the polypeptide encoded by said polynucleotide.
26. A method of altering transport of peptides from the cytosol to the endoplasmic reticulum of a living cell comprising: transfecting a living cell with a vector according to Claim 8, then culturing the transfected cell under conditions suitable to produce the polypeptide encoded by said polynucleotide.
27. A method of altering transport of peptides from the cytosol to the endoplasmic reticulum of a living cell comprising: transfecting a living cell with a vector according to Claim 9, then culturing the transfected cell under conditions suitable to produce the polypeptide encoded by said polynucleotide.
28. A method of altering transport of peptides from the cytosol to the endoplasmic reticulum of a living cell comprising: transfecting a living cell with a vector according to Claim 10, then culturing the transfected cell under conditions suitable to produce the polypeptide encoded by said polynucleotide.
29. A method of altering transport of peptides from the cytosol to the endoplasmic reticulum of a living cell comprising: transfecting a living cell with a vector according to Claim 11, then culturing the transfected cell under conditions suitable to produce the polypeptide encoded by said polynucleotide.
30. A method of altering transport of peptides from the cytosol to the endoplasmic reticulum of a living cell comprising: transfecting a living cell with a vector according to Claim 12, then culturing the transfected cell under conditions suitable to produce the polypeptide encoded by said polynucleotide.

31. A TAP1 splice variant having the ability to form a heterodimer in a mammalian cell with TAP2 (SEQ ID NO: 15), which heterodimer functions as a transporter associated with antigen processing protein.
32. A TAP2 splice variant having the ability to form a heterodimer in a mammalian cell with TAP1 (SEQ ID NO: 19), which heterodimer functions as a transporter associated with antigen processing protein.
33. A TAP2iso exon 12 polypeptide.
34. A TAP2iso exon 12 polypeptide having the sequence of SEQ ID NO: 1.
35. A TAP1iso^3 exon 8a polypeptide.
36. A TAP1iso^3 exon 8a polypeptide comprising the sequence of amino acid residues 582-586 of SEQ ID NO: 25.
37. An isolated antibody reactive with TAP1iso, TAP1iso^2, TAP1iso^3, TAP2iso or TAP2iso^2 but not reactive with TAP1 (SEQ ID NO: 19) or TAP2 (SEQ ID NO: 15) or allelic variants thereof.
38. An isolated antibody according to Claim 37 reactive with TAP2iso exon 12.
39. An isolated antibody according to Claim 37 reactive with TAP1iso^3 exon 8a.
40. A method for treating a disorder associated with abnormal expression of one or more TAP heterodimers comprising gene therapy to provide normal TAP heterodimer expression.
41. A method for broadening the immune response of an individual to a particular antigen comprising (a) removing lymphocytes from the individual, (b) determining whether any TAP isoform is not expressed or is inadequately expressed by the individual, (c) transfecting the lymphocytes with an isolated nucleic acid vector suitable for directing the expression by said lymphocytes of at least one of the non-expressed or inadequately expressed TAP isoforms, and (d) reintroducing the transfected lymphocytes into the individual.
42. A method for diagnosing or monitoring the course of a disease associated with abnormal or inadequate expression of a TAP isoform in an individual, comprising (a) removing a sample of MHC class I-presenting cells from the individual and (b) determining whether such cells exhibit inadequate or abnormal expression of a TAP isoform in comparison to an individual not suffering from said disease, wherein determination of such inadequate expression or abnormal expression of said TAP isoform indicates manifestation of the disease.

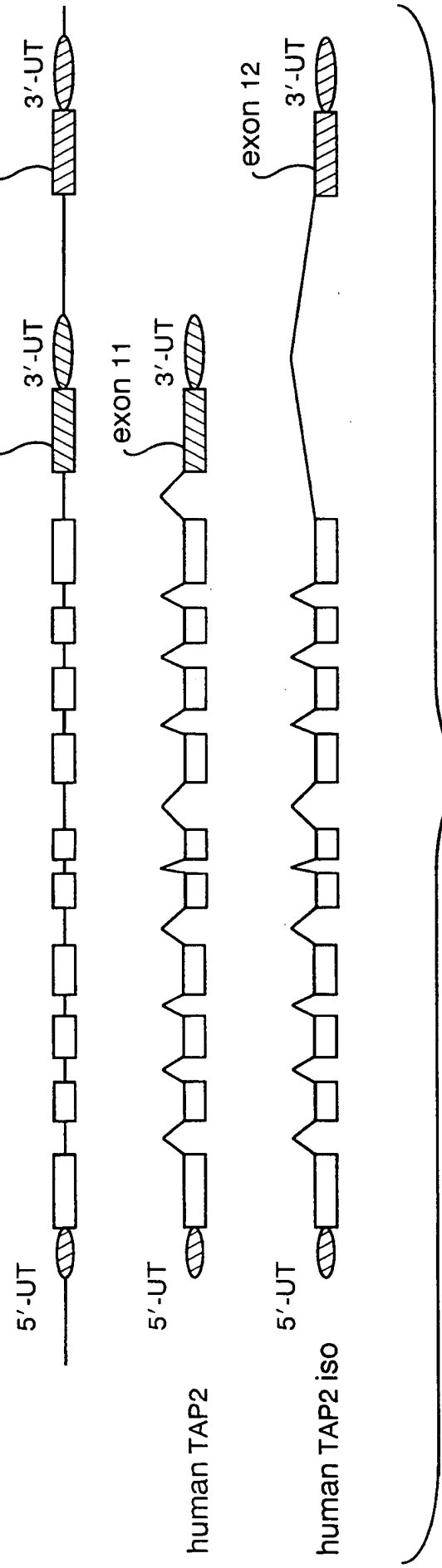

exon 12
3'-UT
exon 11
3'-UT
5'-UT
human TAP2
exon 12
3'-UT
exon 11
3'-UT
5'-UT
human TAP2 iso

FIG. 1

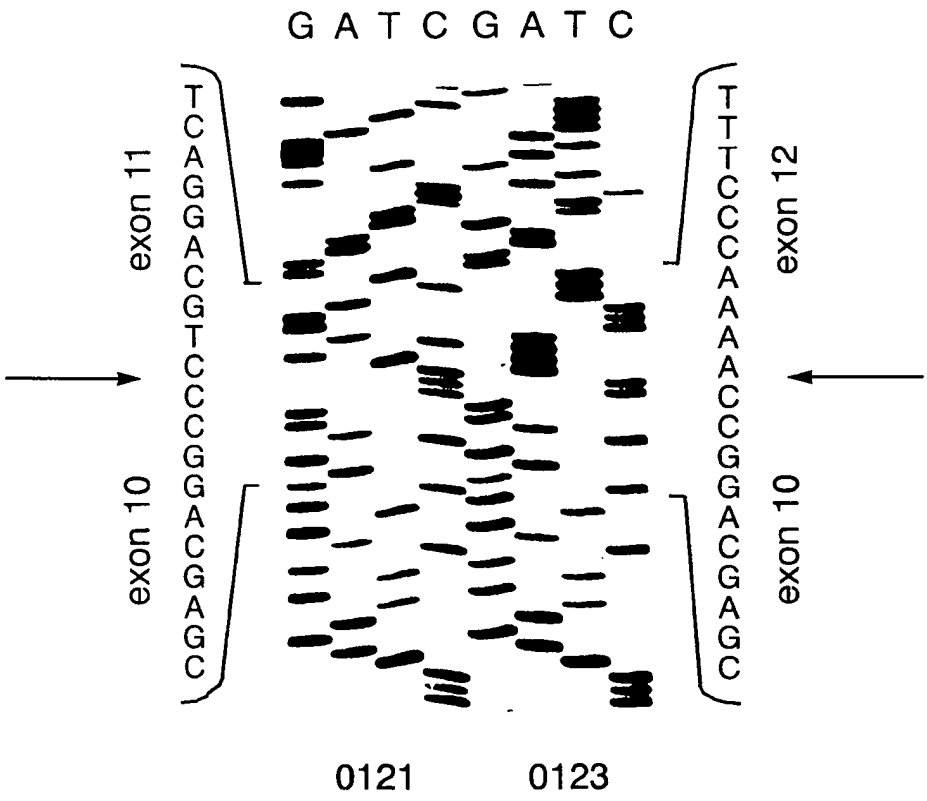

G A T C G A T C
exon 11
T C A G G A C G T C C C G G A C G A G C
exon 10
T T T C C C A A A A C C G G A C G A G C
exon 12
exon 10
0121
0123

FIG. 2

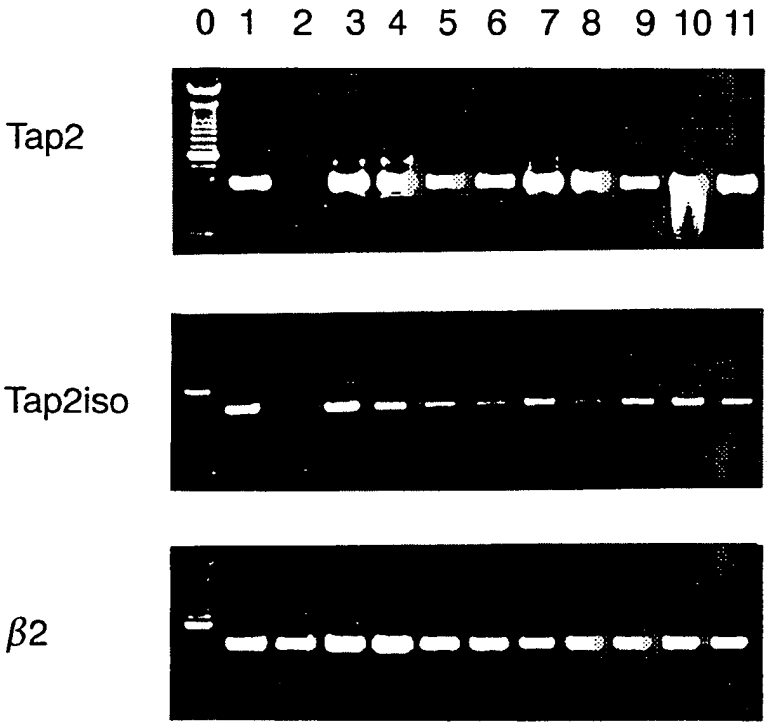
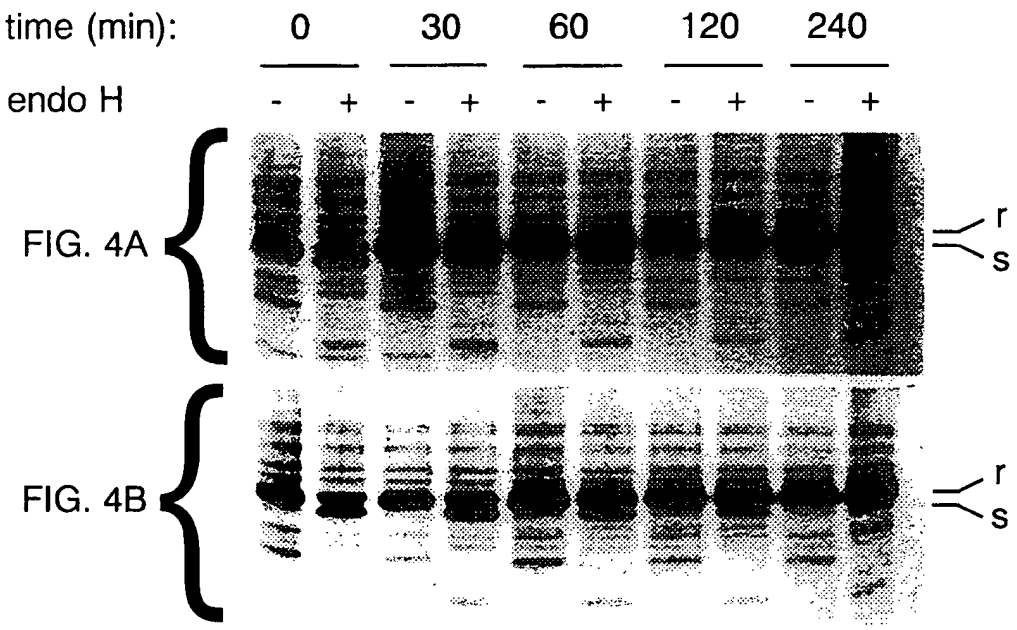


FIG. 3


time (min):
0
30
60
120
240
endo H
- + - + - + - + - +
FIG. 4A
r
s
FIG. 4B
r
s

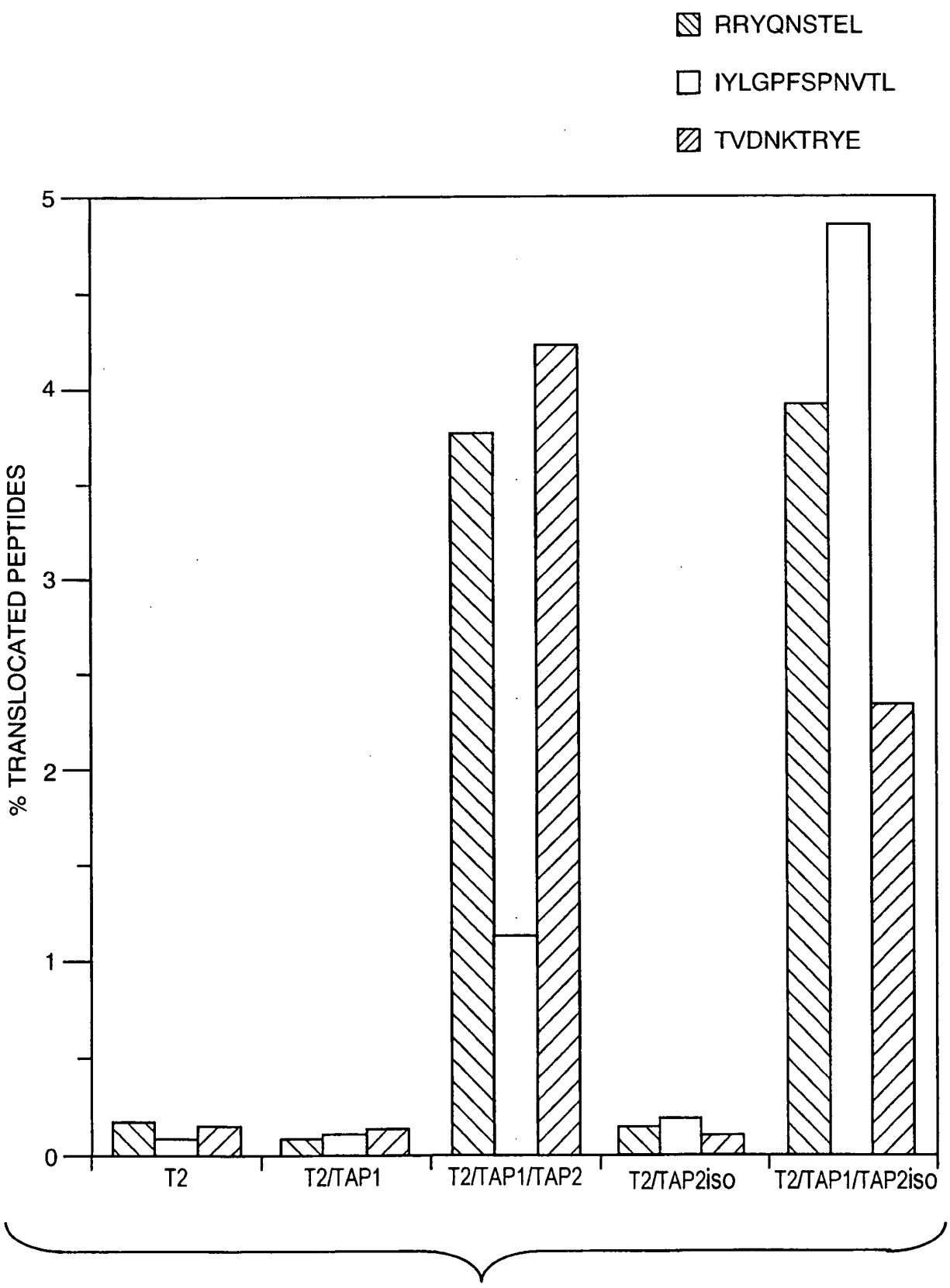
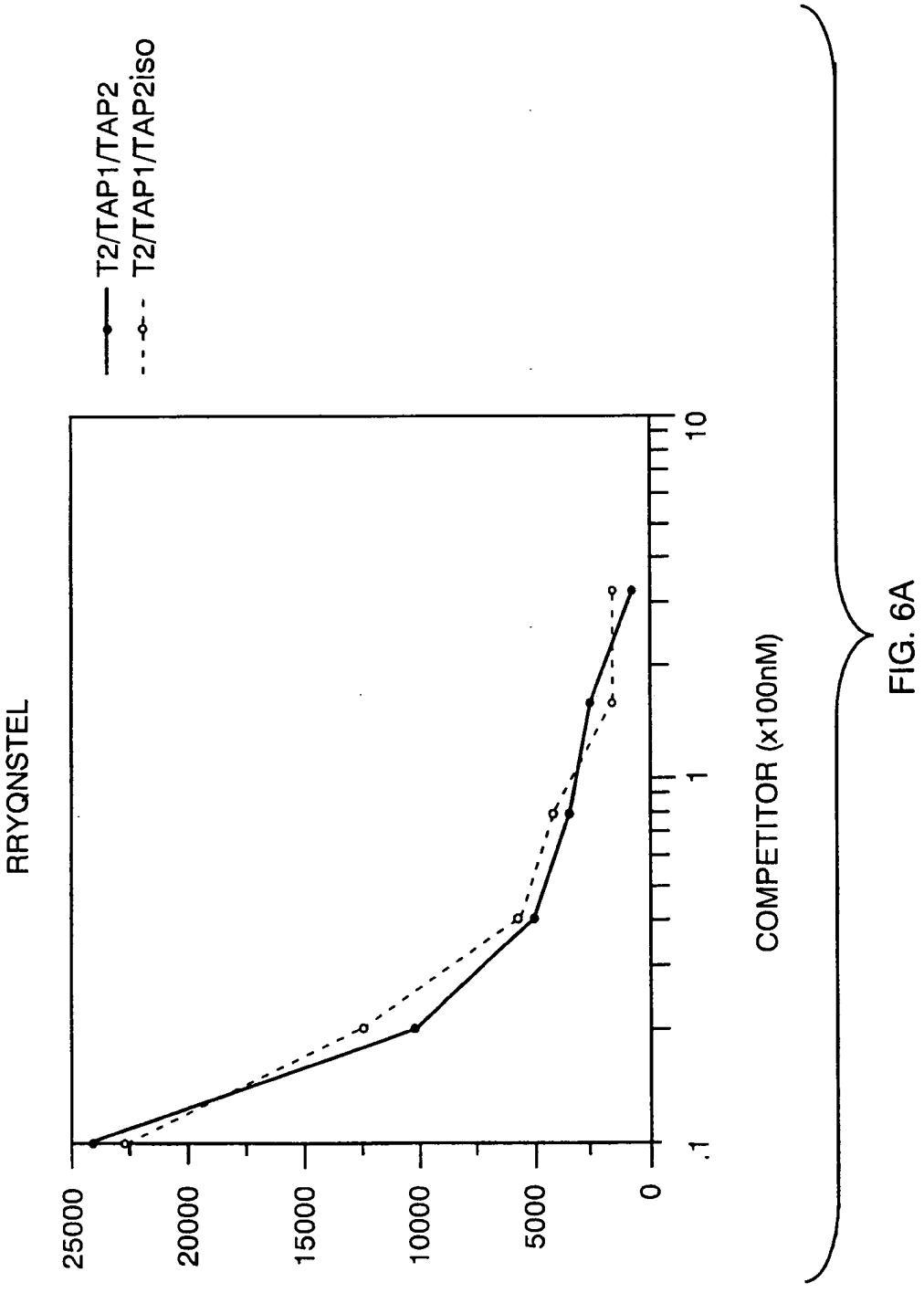

RRYQNSTEL
IYLGPFSPNVTL
TVDNKTRYE
% TRANSLOCATED PEPTIDES
0
1
2
3
4
5
T2
T2/TAP1
T2/TAP1/TAP2
T2/TAP2iso
T2/TAP1/TAP2iso

FIG. 5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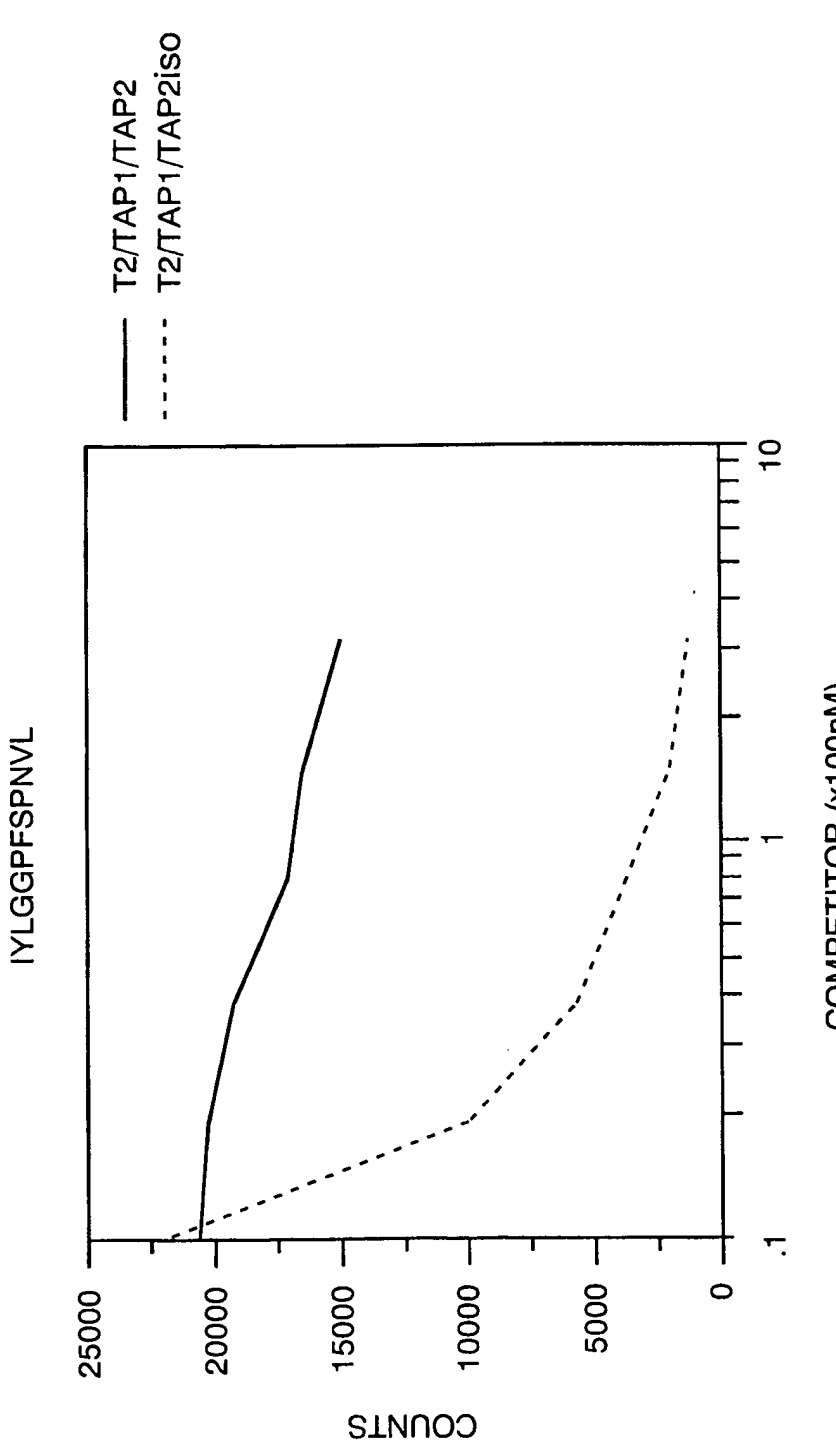
COMPETITOR (x100nM)

FIG. 6A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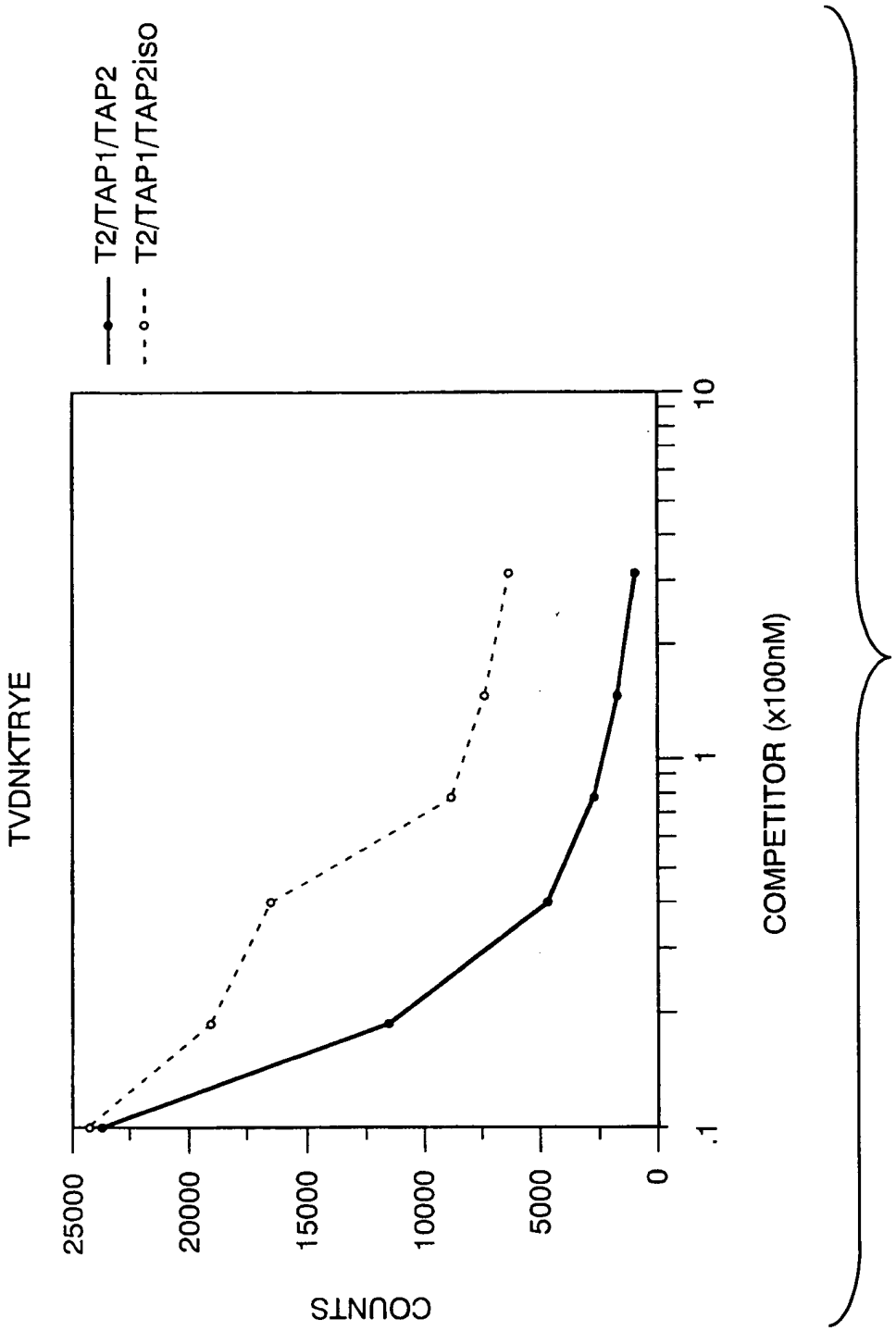
10
COMPETITOR (x100nM)

FIG. 6B


T2/TAP1/TAP2
T2/TAP1/TAP2iso
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COUNTS
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20000
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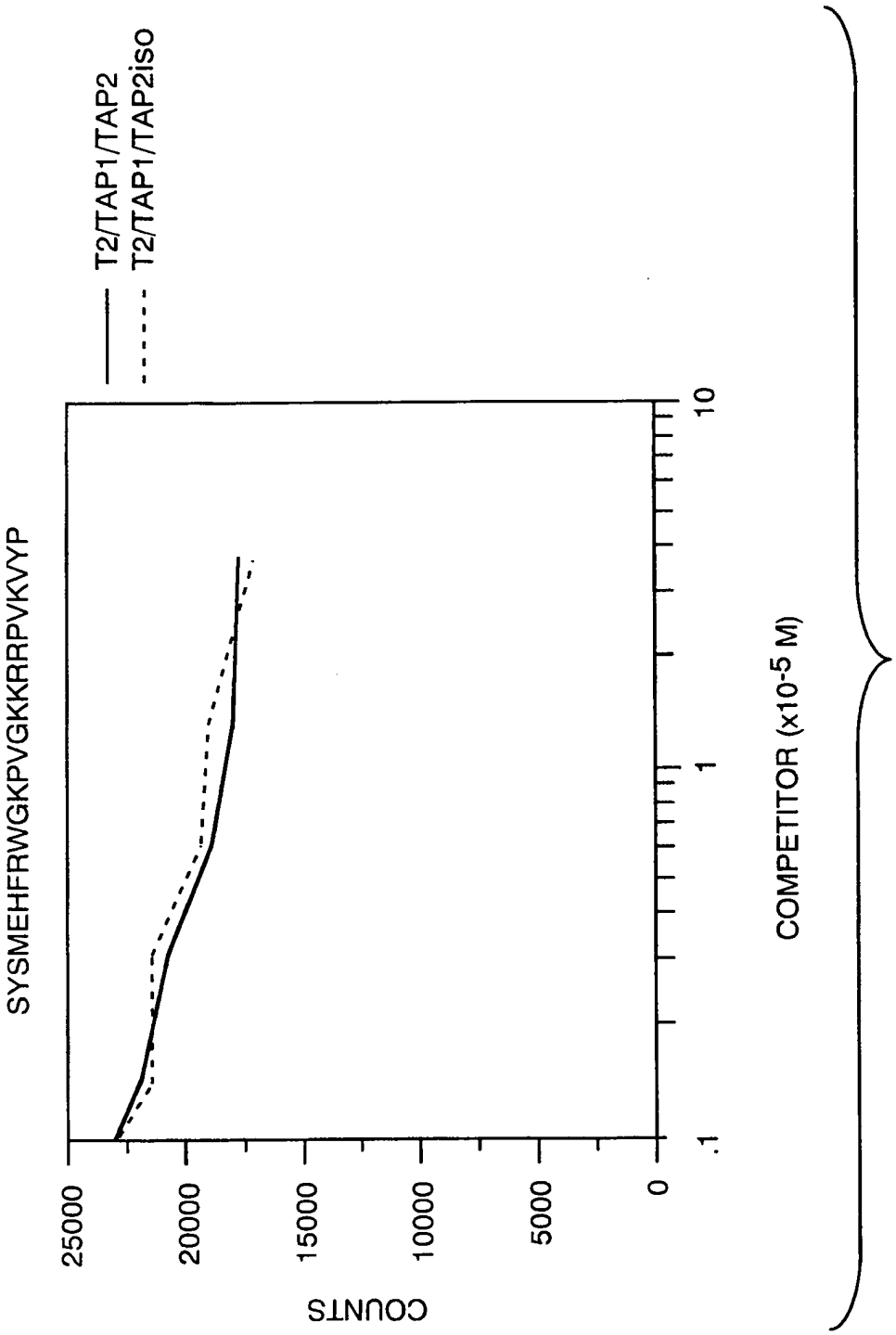
0
.1
1
10
COMPETITOR (x100nM)

FIG. 6C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T2/TAP1/TAP2iso
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COUNTS
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10000
5000
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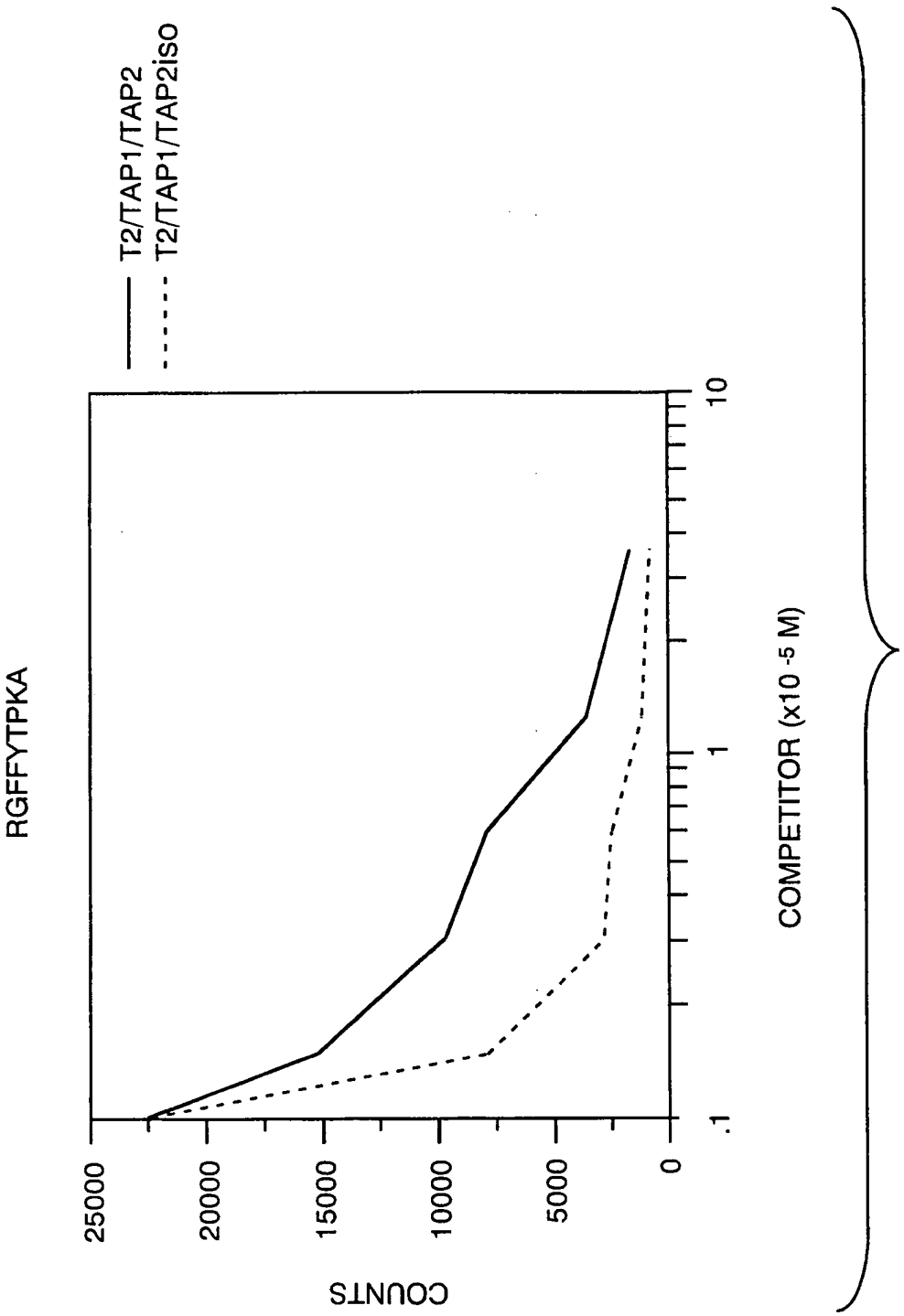
1
10
COMPETITOR (x10-5 M)

FIG. 6D


T2/TAP1/TAP2
T2/TAP1/TAP2iso
RGFFYTPKA
COUNTS
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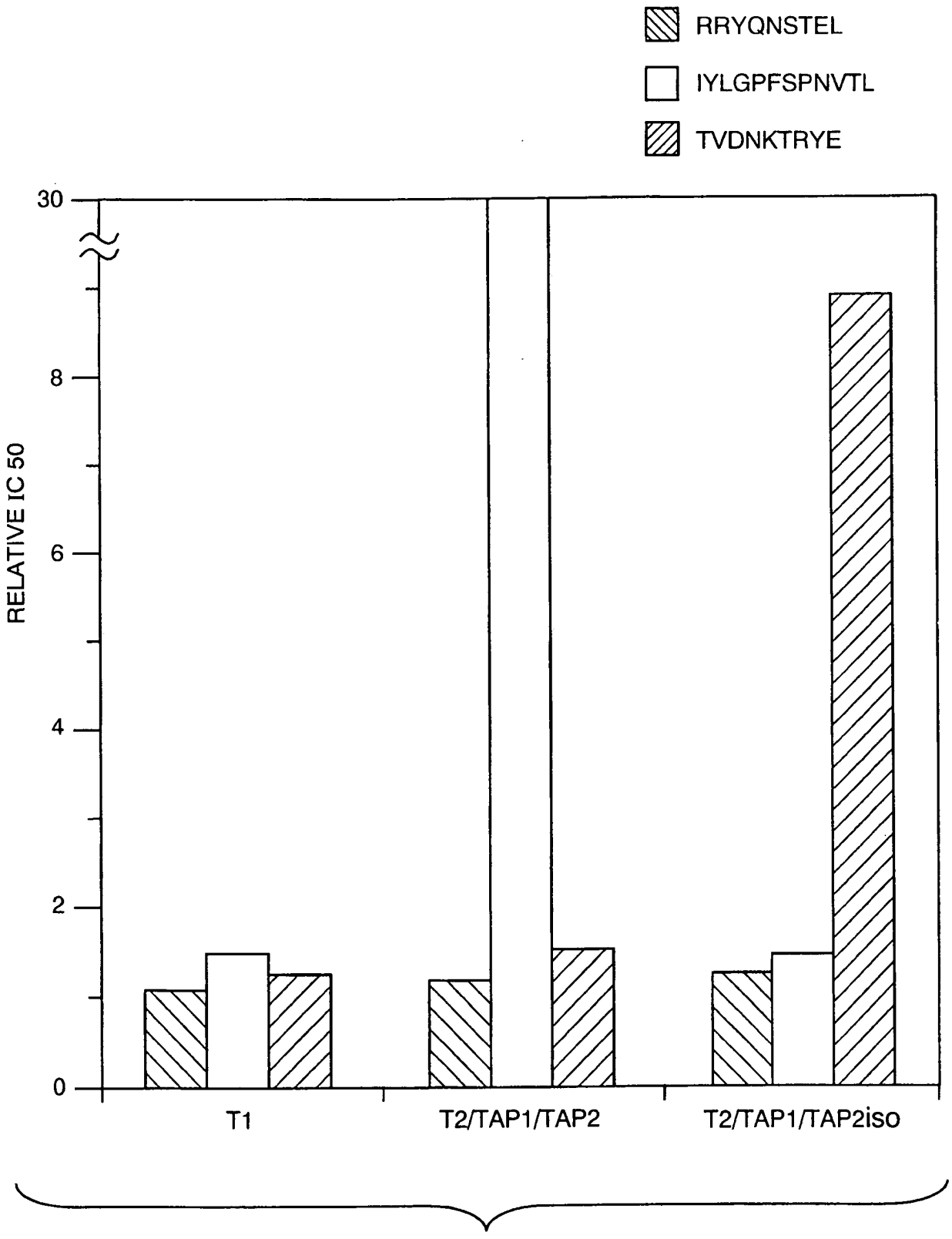
.1
1
10
COMPETITOR (x10 -5 M)

FIG. 6E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IYLGPFSPNVTL
TVDNKTRYE
RELATIVE IC 50
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8
6
4
2
0
T1
T2/TAP1/TAP2
T2/TAP1/TAP2iso

FIG. 7

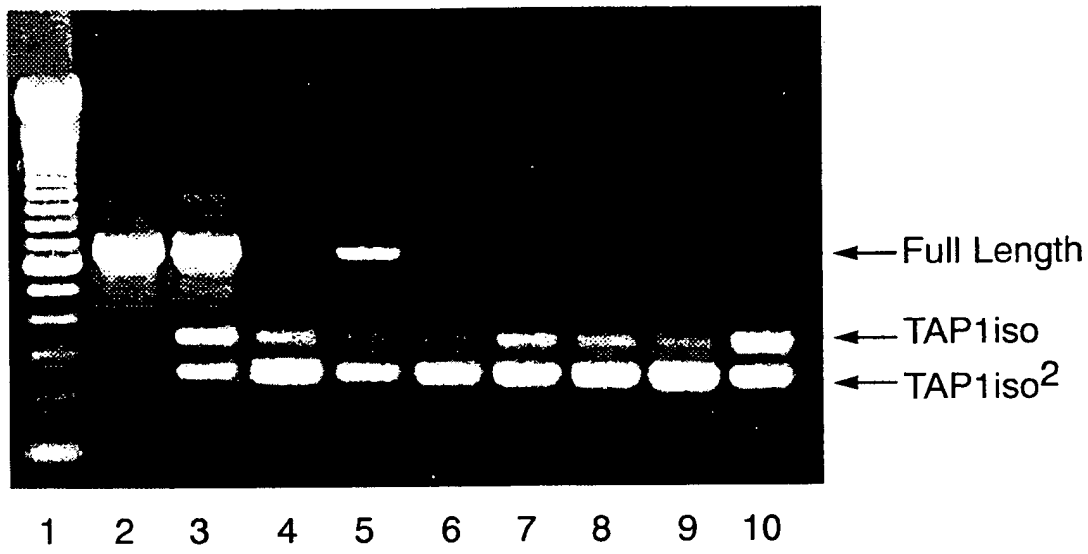
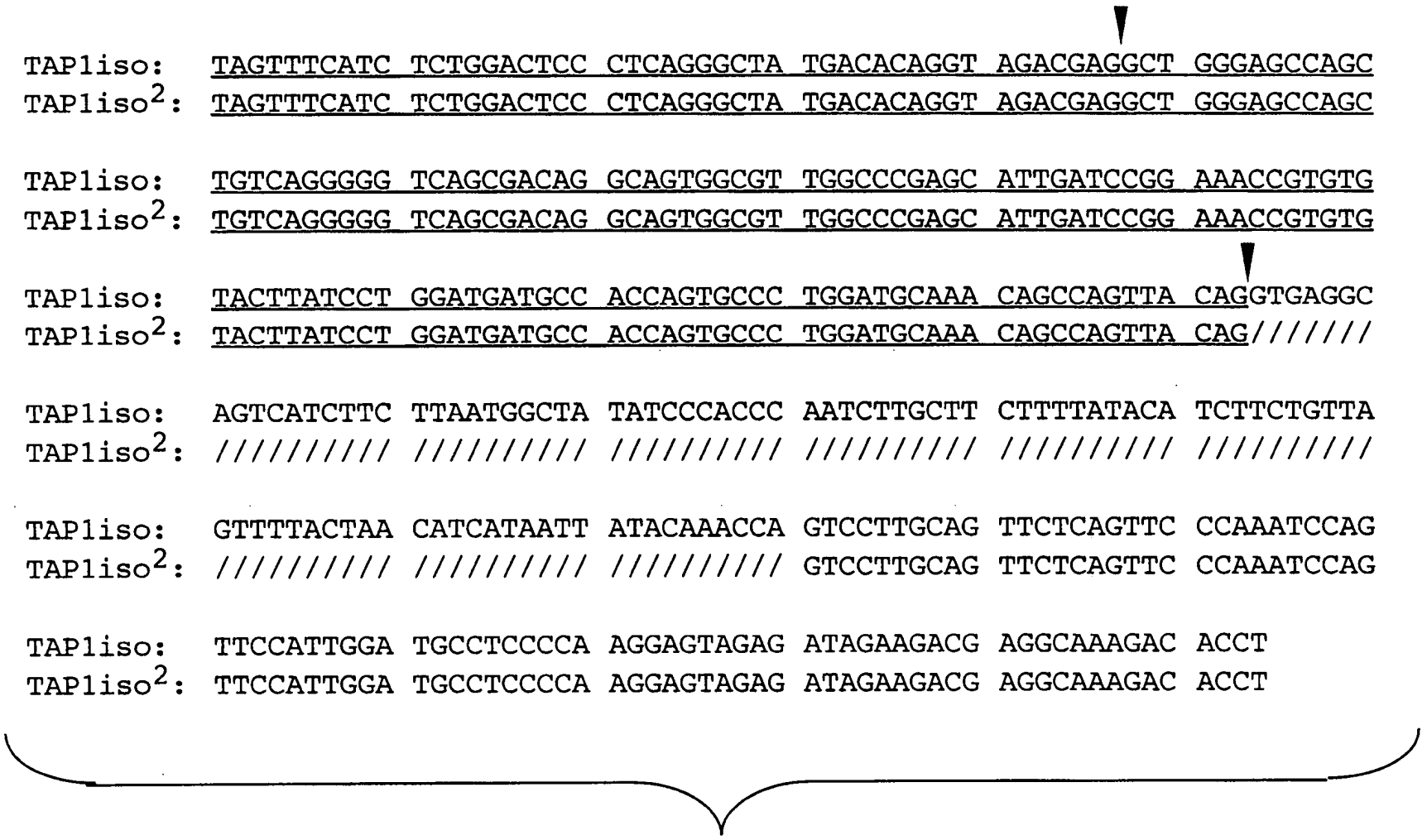


FIG. 8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TAP1iso: TGTCAGGGGG TCAGCGACAG GCAGTGGCGT TGGCCCGAGC ATTGATCCGG AAACCGTGTG
TAP1iso2: TGTCAGGGGG TCAGCGACAG GCAGTGGCGT TGGCCCGAGC ATTGATCCGG AAACCGTGTG
TAP1iso: TACTTATCCT GGATGATGCC ACCAGTGCCC TGGATGCAAA CAGCCAGTTA CAGGTGAGGC
TAP1iso2: TACTTATCCT GGATGATGCC ACCAGTGCCC TGGATGCAAA CAGCCAGTTA CAG///////
TAP1iso: AGTCATCTTC TTAATGGCTA TATCCCACCC AATCTTGCTT CTTTTATACA TCTTCTGTTA
TAP1iso2: ////////// ////////// ////////// ////////// ////////// //////////
TAP1iso: GTTTTACTAA CATCATAATT ATACAAACCA GTCCTTGCAG TTCTCAGTTC CCAAATCCAG
TAP1iso2: ////////// ////////// ////////// GTCCTTGCAG TTCTCAGTTC CCAAATCCAG
TAP1iso: TTCCATTGGA TGCCTCCCCA AGGAGTAGAG ATAGAAGACG AGGCAAAGAC ACCT
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FIG. 9A

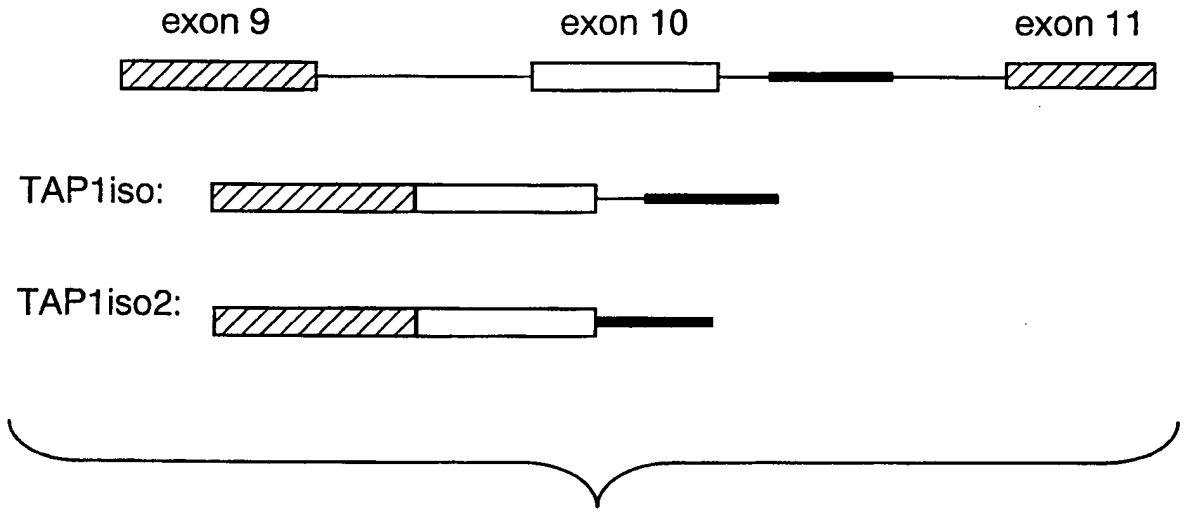


FIG. 9B

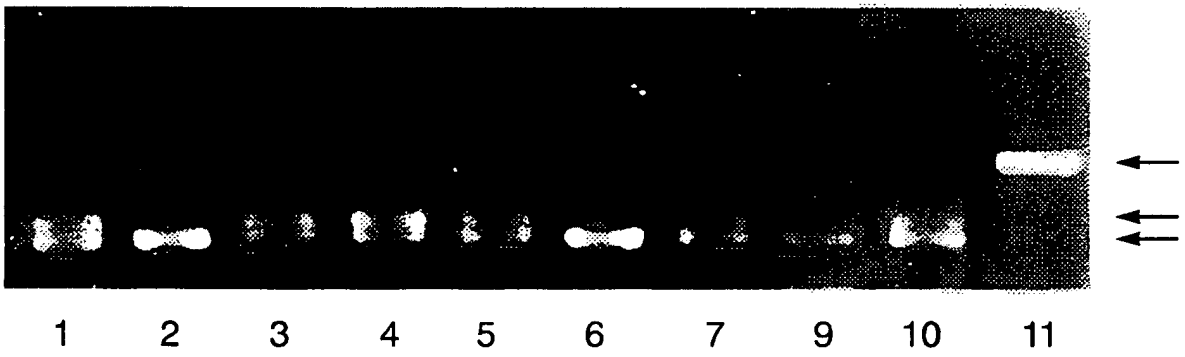
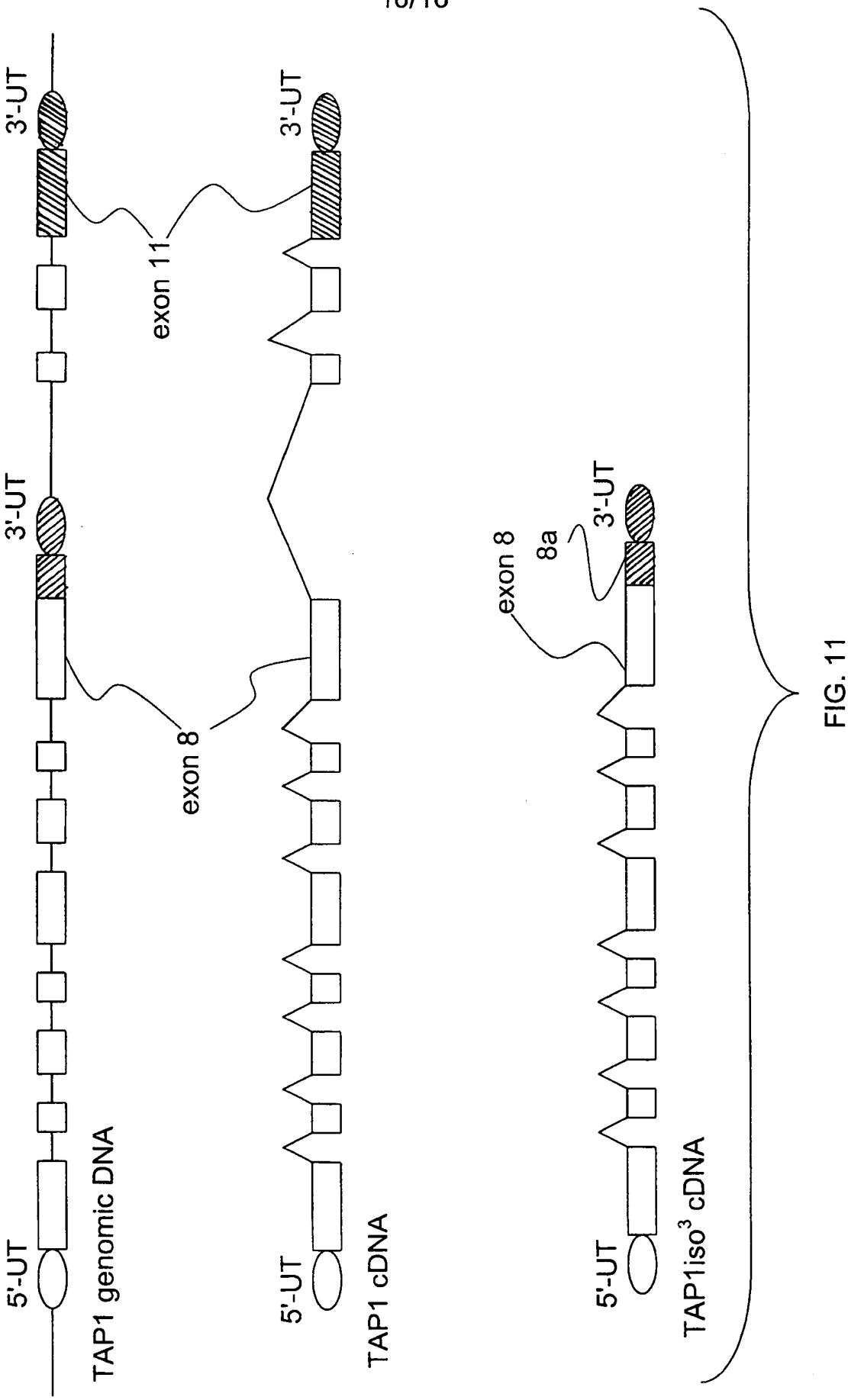


FIG. 10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exon 11
3'-UT
3'-UT
exon 8
5'-UT
TAP1 genomic DNA
5'-UT
TAP1 cDNA
exon 8
8a
3'-UT
5'-UT
TAP1iso3 cDNA

FIG. 11

SEQUENCE LISTING

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The General Hospital Corporation

<120> TRANSPORTER PROTEIN SPLICE VARIANTS AND MODEL FOR IMMUNE DIVERSITY

<130> seqlist-MGH-002.1 PCT (MGH-1247-1)

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Gln Gly Leu Pro Gly Leu Trp Leu Glu Gly Thr Leu Arg Leu Gly Gly
35 40 45

Leu Trp Gly Leu Leu Lys Leu Arg Gly Leu Leu Gly Phe Val Gly Thr
50 55 60

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                85                  90                  95

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            100                 105                 110

Trp Ser Leu Trp Ala Val Leu Ser Pro Pro Gly Ala Gln Glu Lys Glu
        115                 120                 125

Gln Asp Gln Val Asn Asn Lys Val Leu Met Trp Arg Leu Leu Lys Leu
    130                 135                 140

Ser Arg Pro Asp Leu Pro Leu Leu Val Ala Ala Phe Phe Phe Leu Val
145                 150                 155                 160

Leu Ala Val Leu Gly Glu Thr Leu Ile Pro His Tyr Ser Gly Arg Val
                165                 170                 175

Ile Asp Ile Leu Gly Gly Asp Phe Asp Pro His Ala Phe Ala Ser Ala
            180                 185                 190

Ile Phe Phe Met Cys Leu Phe Ser Phe Gly Ser Ser Leu Ser Ala Gly
        195                 200                 205

Cys Arg Gly Gly Cys Phe Thr Tyr Thr Met Ser Arg Ile Asn Leu Arg
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Ile Arg Glu Gln Leu Phe Ser Ser Leu Leu Arg Gln Asp Leu Gly Phe
225                 230                 235                 240

Phe Gln Glu Thr Lys Thr Gly Glu Leu Asn Ser Arg Leu Ser Ser Asp
                245                 250                 255

Thr Thr Leu Met Ser Asn Trp Leu Pro Leu Asn Ala Asn Val Leu Leu
            260                 265                 270

Arg Ser Leu Val Lys Val Val Gly Leu Tyr Gly Phe Met Leu Ser Ile
        275                 280                 285

Ser Pro Arg Leu Thr Leu Leu Ser Leu Leu His Met Pro Phe Thr Ile
    290                 295                 300

Ala Ala Glu Lys Val Tyr Asn Thr Arg His Gln Glu Val Leu Arg Glu
305                 310                 315                 320
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Ile Gln Asp Ala Val Ala Arg Ala Gly Gln Val Val Arg Glu Ala Val
                325                 330                 335

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            340                 345                 350

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    370                 375                 380

Leu Gly Val Gln Met Leu Met Leu Ser Cys Gly Leu Gln Gln Met Gln
385                 390                 395                 400

Asp Gly Glu Leu Thr Gln Gly Ser Leu Leu Ser Phe Met Ile Tyr Gln
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    450                 455                 460

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465                 470                 475                 480

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                485                 490                 495

Glu Val Thr Ala Leu Val Gly Pro Asn Gly Ser Gly Lys Ser Thr Val
            500                 505                 510

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        515                 520                 525

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    530                 535                 540

Gln Val Val Ser Val Gly Gln Glu Pro Val Leu Phe Ser Gly Ser Val
545                 550                 555                 560

Arg Asn Asn Ile Ala Tyr Gly Leu Gln Ser Cys Glu Asp Asp Lys Val
                565                 570                 575
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595 600 605

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ggaggtgatt ttgaccccca tgcctttgcc agtgccatct tcttcatgtg cctcttctcc 600
tttggcagct cactgtctgc aggctgccga ggaggctgct tcacctacac catgtctcga 660
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tgcgagcagg ccaaaaccct ttggaagttc atgatattt 1959

<210> 5
<211> 27
<212> DNA
<213> Homo sapiens

<400> 5
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<210> 6
<211> 9
<212> PRT
<213> Artificial Sequence

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<223> Description of Artificial Sequence: test peptide
for translocation study

<400> 6
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<210> 7
<211> 12
<212> PRT
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: test peptide
for translocation study

<400> 7
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1 5 10

<210> 8
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: test peptide
for translocation study

<400> 8
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1 5

<210> 9
<211> 24
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: test peptide
for translocation study

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Ser Tyr Ser Met Glu His Phe Arg Trp Gly Lys Pro Val Gly Lys Lys
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Arg Arg Pro Val Lys Val Tyr Pro
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<210> 10
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: insulin beta
chain amino acids 22-30

<400> 10
Arg Gly Phe Phe Tyr Thr Pro Lys Ala
1 5

<210> 11
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide probe

<400> 11
gtcctggaga cgcccctgag aagag 25

<210> 12
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide probe

<400> 12
atcccaccta gtgagaatcc 20

<210> 13
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide probe

<400> 13
cattcctgaa gctgacagca 20

<210> 14
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:

oligonucleotide probe

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ttcaacctcc atgatgctgc 20

<210> 15
<211> 686
<212> PRT
<213> Homo sapiens

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Ala Ala Leu Leu Trp Leu Leu Gln Gly Pro Leu Gly Thr Leu Leu Pro
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Gln Gly Leu Pro Gly Leu Trp Leu Glu Gly Thr Leu Arg Leu Gly Gly
35 40 45

Leu Trp Gly Leu Leu Lys Leu Arg Gly Leu Leu Gly Phe Val Gly Thr
50 55 60

Leu Leu Leu Pro Leu Cys Leu Ala Thr Pro Leu Thr Val Ser Leu Arg
65 70 75 80

Ala Leu Val Ala Gly Ala Ser Arg Ala Pro Pro Ala Arg Val Ala Ser
85 90 95

Ala Pro Trp Ser Trp Leu Leu Val Gly Tyr Gly Ala Ala Gly Leu Ser
100 105 110

Trp Ser Leu Trp Ala Val Leu Ser Pro Pro Gly Ala Gln Glu Lys Glu
115 120 125

Gln Asp Gln Val Asn Asn Lys Val Leu Met Trp Arg Leu Leu Lys Leu
130 135 140

Ser Arg Pro Asp Leu Pro Leu Leu Val Ala Ala Phe Phe Phe Leu Val
145 150 155 160

Leu Ala Val Leu Gly Glu Thr Leu Ile Pro His Tyr Ser Gly Arg Val
165 170 175

Ile Asp Ile Leu Gly Gly Asp Phe Asp Pro His Ala Phe Ala Ser Ala
180 185 190

Ile Phe Phe Met Cys Leu Phe Ser Phe Gly Ser Ser Leu Ser Ala Gly

195 200 205

Cys Arg Gly Gly Cys Phe Thr Tyr Thr Met Ser Arg Ile Asn Leu Arg
210 215 220

Ile Arg Glu Gln Leu Phe Ser Ser Leu Leu Arg Gln Asp Leu Gly Phe
225 230 235 240

Phe Gln Glu Thr Lys Thr Gly Glu Leu Asn Ser Arg Leu Ser Ser Asp
245 250 255

Thr Thr Leu Met Ser Asn Trp Leu Pro Leu Asn Ala Asn Val Leu Leu
260 265 270

Arg Ser Leu Val Lys Val Val Gly Leu Tyr Gly Phe Met Leu Ser Ile
275 280 285

Ser Pro Arg Leu Thr Leu Leu Ser Leu Leu His Met Pro Phe Thr Ile
290 295 300

Ala Ala Glu Lys Val Tyr Asn Thr Arg His Gln Glu Val Leu Arg Glu
305 310 315 320

Ile Gln Asp Ala Val Ala Arg Ala Gly Gln Val Val Arg Glu Ala Val
325 330 335

Gly Gly Leu Gln Thr Val Arg Ser Phe Gly Ala Glu Glu His Glu Val
340 345 350

Cys Arg Tyr Lys Glu Ala Lys Glu Gln Cys Arg Gln Leu Tyr Trp Arg
355 360 365

Arg Asp Leu Glu Arg Ala Leu Tyr Leu Leu Ile Arg Arg Val Leu His
370 375 380

Leu Gly Val Gln Met Leu Met Leu Ser Cys Gly Leu Gln Gln Met Gln
385 390 395 400

Asp Gly Glu Leu Thr Gln Gly Ser Leu Leu Ser Phe Met Ile Tyr Gln
405 410 415

Glu Ser Val Gly Ser Tyr Val Gln Thr Leu Val Tyr Ile Tyr Gly Asp
420 425 430

Met Leu Ser Asn Val Gly Ala Ala Glu Lys Val Phe Ser Tyr Met Asp
435 440 445

Arg Gln Pro Asn Leu Pro Ser Pro Gly Thr Leu Ala Pro Thr Thr Leu

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Gln Gly Val Val Lys Phe Gln Asp Val Ser Phe Ala Tyr Pro Asn Arg
465                 470                 475                 480

Pro Asp Arg Pro Val Leu Lys Gly Leu Thr Phe Thr Leu Arg Pro Gly
                485                 490                 495

Glu Val Thr Ala Leu Val Gly Pro Asn Gly Ser Gly Lys Ser Thr Val
            500                 505                 510

Ala Ala Leu Leu Gln Asn Leu Tyr Gln Pro Thr Gly Gly Gln Val Leu
        515                 520                 525

Leu Asp Glu Lys Pro Ile Ser Gln Tyr Glu His Cys Tyr Leu His Ser
    530                 535                 540

Gln Val Val Ser Val Gly Gln Glu Pro Val Leu Phe Ser Gly Ser Val
545                 550                 555                 560

Arg Asn Asn Ile Ala Tyr Gly Leu Gln Ser Cys Glu Asp Asp Lys Val
                565                 570                 575

Met Ala Ala Ala Gln Ala Ala His Ala Asp Asp Phe Ile Gln Glu Met
            580                 585                 590

Glu His Gly Ile Tyr Thr Asp Val Gly Glu Lys Gly Ser Gln Leu Ala
        595                 600                 605

Ala Gly Gln Lys Gln Arg Leu Ala Ile Ala Arg Ala Leu Val Arg Asp
    610                 615                 620

Pro Arg Val Leu Ile Leu Asp Glu Ala Thr Ser Ala Leu Asp Val Gln
625                 630                 635                 640

Cys Glu Gln Ala Leu Gln Asp Trp Asn Ser Arg Gly Asp Arg Thr Val
                645                 650                 655

Leu Val Ile Ala His Arg Leu Gln Thr Val Gln Arg Ala His Gln Ile
            660                 665                 670

Leu Val Leu Gln Glu Gly Lys Leu Gln Lys Leu Ala Gln Leu
        675                 680                 685
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<210> 16
<211> 42
<212> PRT

<213> Homo sapiens

<400> 16

Leu Gln Asp Trp Asn Ser Arg Gly Asp Arg Thr Val Leu Val Ile Ala
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His Arg Leu Gln Thr Val Gln Arg Ala His Gln Ile Leu Val Leu Gln
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Glu Gly Lys Leu Gln Lys Leu Ala Gln Leu
35 40

<210> 17
<211> 2061
<212> DNA
<213> Homo sapiens

<400> 17

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<210> 18
<211> 2244
<212> DNA
<213> Homo sapiens

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<210> 19
<211> 748
<212> PRT
<213> Homo sapiens

<400> 19

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Gly Ala Ser Leu Ala Trp Leu Gly Thr Val Leu Leu Leu Leu Ala Asp
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Trp Val Leu Leu Arg Thr Ala Leu Pro Arg Ile Phe Ser Leu Leu Val
         35                  40                  45

Pro Thr Ala Leu Pro Leu Leu Arg Val Trp Ala Val Gly Leu Ser Arg
     50                  55                  60

Trp Ala Val Leu Trp Leu Gly Ala Cys Gly Val Leu Arg Ala Thr Val
 65                  70                  75                  80

Gly Ser Lys Ser Glu Asn Ala Gly Ala Gln Gly Trp Leu Ala Ala Leu
                 85                  90                  95

Lys Pro Leu Ala Ala Ala Leu Gly Leu Ala Leu Pro Gly Leu Ala Leu
            100                 105                 110

Phe Arg Glu Leu Ile Ser Trp Gly Ala Pro Gly Ser Ala Asp Ser Thr
        115                 120                 125

Arg Leu Leu His Trp Gly Ser His Pro Thr Ala Phe Val Val Ser Tyr
    130                 135                 140

Ala Ala Ala Leu Pro Ala Ala Ala Leu Trp His Lys Leu Gly Ser Leu
145                 150                 155                 160

Trp Val Pro Gly Gly Gln Gly Gly Ser Gly Asn Pro Val Arg Arg Leu
                165                 170                 175

Leu Gly Cys Leu Gly Ser Glu Thr Arg Arg Leu Ser Leu Phe Leu Val
            180                 185                 190

Leu Val Val Leu Ser Ser Leu Gly Glu Met Ala Ile Pro Phe Phe Thr
        195                 200                 205
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Gly Arg Leu Thr Asp Trp Ile Leu Gln Asp Gly Ser Ala Asp Thr Phe
    210                 215                 220

Thr Arg Asn Leu Thr Leu Met Ser Ile Leu Thr Ile Ala Ser Ala Val
225                 230                 235                 240

Leu Glu Phe Val Gly Asp Gly Ile Tyr Asn Asn Thr Met Gly His Val
                245                 250                 255

His Ser His Leu Gln Gly Glu Val Phe Gly Ala Val Leu Arg Gln Glu
            260                 265                 270

Thr Glu Phe Phe Gln Gln Asn Gln Thr Gly Asn Ile Met Ser Arg Val
        275                 280                 285

Thr Glu Asp Thr Ser Thr Leu Ser Asp Ser Leu Ser Glu Asn Leu Ser
    290                 295                 300

Leu Phe Leu Trp Tyr Leu Val Arg Gly Leu Cys Leu Leu Gly Ile Met
305                 310                 315                 320

Leu Trp Gly Ser Val Ser Leu Thr Met Val Thr Leu Ile Thr Leu Pro
                325                 330                 335

Leu Leu Phe Leu Leu Pro Lys Lys Val Gly Lys Trp Tyr Gln Leu Leu
            340                 345                 350

Glu Val Gln Val Arg Glu Ser Leu Ala Lys Ser Ser Gln Val Ala Ile
        355                 360                 365

Glu Ala Leu Ser Ala Met Pro Thr Val Arg Ser Phe Ala Asn Glu Glu
    370                 375                 380

Gly Glu Ala Gln Lys Phe Arg Glu Lys Leu Gln Glu Ile Lys Thr Leu
385                 390                 395                 400

Asn Gln Lys Glu Ala Val Ala Tyr Ala Val Asn Ser Trp Thr Thr Ser
                405                 410                 415

Ile Ser Gly Met Leu Leu Lys Val Gly Ile Leu Tyr Ile Gly Gly Gln
            420                 425                 430

Leu Val Thr Ser Gly Ala Val Ser Ser Gly Asn Leu Val Thr Phe Val
        435                 440                 445

Leu Tyr Gln Met Gln Phe Thr Gln Ala Val Glu Val Leu Leu Ser Ile
    450                 455                 460
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Tyr Pro Arg Val Gln Lys Ala Val Gly Ser Ser Glu Lys Ile Phe Glu
465                 470                 475                 480

Tyr Leu Asp Arg Thr Pro Arg Cys Pro Pro Ser Gly Leu Leu Thr Pro
                485                 490                 495

Leu His Leu Glu Gly Leu Val Gln Phe Gln Asp Val Ser Phe Ala Tyr
            500                 505                 510

Pro Asn Arg Pro Asp Val Leu Val Leu Gln Gly Leu Thr Phe Thr Leu
        515                 520                 525

Arg Pro Gly Glu Val Thr Ala Leu Val Gly Pro Asn Gly Ser Gly Lys
    530                 535                 540

Ser Thr Val Ala Ala Leu Leu Gln Asn Leu Tyr Gln Pro Thr Gly Gly
545                 550                 555                 560

Gln Leu Leu Leu Asp Gly Lys Pro Leu Pro Gln Tyr Glu His Arg Tyr
                565                 570                 575

Leu His Arg Gln Val Ala Ala Val Gly Gln Glu Pro Gln Val Phe Gly
            580                 585                 590

Arg Ser Leu Gln Glu Asn Ile Ala Tyr Gly Leu Thr Gln Lys Pro Thr
        595                 600                 605

Met Glu Glu Ile Thr Ala Ala Ala Val Lys Ser Gly Ala His Ser Phe
    610                 615                 620

Ile Ser Gly Leu Pro Gln Gly Tyr Asp Thr Glu Val Asp Glu Ala Gly
625                 630                 635                 640

Ser Gln Leu Ser Gly Gly Gln Arg Gln Ala Val Ala Leu Ala Arg Ala
                645                 650                 655

Leu Ile Arg Lys Pro Cys Val Leu Ile Leu Asp Asp Ala Thr Ser Ala
            660                 665                 670

Leu Asp Ala Asn Ser Gln Leu Gln Val Glu Gln Leu Leu Tyr Glu Ser
        675                 680                 685

Pro Glu Arg Tyr Ser Arg Ser Val Leu Leu Ile Thr Gln His Leu Ser
    690                 695                 700

Leu Val Glu Gln Ala Asp His Ile Leu Phe Leu Glu Gly Gly Ala Ile
705                 710                 715                 720
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Arg Glu Gly Gly Thr His Gln Gln Leu Met Glu Lys Lys Gly Cys Tyr
725 730 735

Trp Ala Met Val Gln Ala Pro Ala Asp Ala Pro Glu
740 745

<210> 20
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer

<400> 20
tagtttcatc tctggactcc ctca 24

<210> 21
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer

<400> 21
aggtgtcttt gcctcgtctt ct 22

<210> 22
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer

<400> 22
gctactagtg ccctagatgt gcagt 25

<210> 23
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer

<400> 23
cttctgcagc ttgccctcct ggag 24

<210> 24
<211> 1977
<212> DNA
<213> Homo sapiens

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Pro Thr Ala Leu Pro Leu Leu Arg Val Trp Ala Val Gly Leu Ser Arg
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Trp Ala Val Leu Trp Leu Gly Ala Cys Gly Val Leu Arg Ala Thr Val
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Gly Ser Lys Ser Glu Asn Ala Gly Ala Gln Gly Trp Leu Ala Ala Leu
                 85                  90                  95

Lys Pro Leu Ala Ala Ala Leu Gly Leu Ala Leu Pro Gly Leu Ala Leu
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Phe Arg Glu Leu Ile Ser Trp Gly Ala Pro Gly Ser Ala Asp Ser Thr
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Arg Leu Leu His Trp Gly Ser His Pro Thr Ala Phe Val Val Ser Tyr
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Ala Ala Ala Leu Pro Ala Ala Ala Leu Trp His Lys Leu Gly Ser Leu
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Trp Val Pro Gly Gly Gln Gly Gly Ser Gly Asn Pro Val Arg Arg Leu
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Leu Gly Cys Leu Gly Ser Glu Thr Arg Arg Leu Ser Leu Phe Leu Val
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His Ser His Leu Gln Gly Glu Val Phe Gly Ala Val Leu Arg Gln Glu
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08205

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :536/23.1; 435/320.1, 325, 252.3; 530/300, 350, 387.1; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/320.1, 325, 252.3; 530/300, 350, 387.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG-Medline, Embase, Scisearch, Biosis, Cancerlit; APS, Derwint
search terms: TAP1, TAP2, splice variants, RING4, RING11

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	**BECK. S.** et al., DNA sequence Analysis of 66 kb of the human mhc class II region encoding a cluster of genes for antigen processing. J. Mol. Biol. 1992. Vol. 228. pages 433-441, especially page 437.	1-6 ----- 7-42
X --- Y	**POWIS. S.H.** et al., Alleles and haplotypes of the MHC-encoded ABC transporters TAP1 and TAP2. Immunogenetics. 1993. Vol. 37. pages 373-380, especially page 378.	1-6 ----- 7-42
X --- Y	**BAHRAM. S.** et al., Two putative subunits of a peptide pump encoded in the human major histocompatibility complex class II region. Proc. Natl. Acad. Sci. USA. November 1991. Vol. 88. pages 10094-10098, especially page10095.	1-6, 31-36 ---------- 7-30, 37-42

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 JULY 1999	Date of mailing of the international search report 23 AUG 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE MARIE S. BECKERLEG Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08205

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92/11289 A1 (**IMPERIAL CANCER RESEARCH TECHNOLOGY LTD.**) 09 July 1992, see entire document.	1-42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08205

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/04; C12N 15/11, 15/63, 15/85, 1/21; C07K 5/00, 14/00, 16/00; A61K 48/00